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From the Department of Physiological Chemistry University of Lund Sweden

The Metabolism of Fatty Acids in the Rat

VI Arachidonic Acid

By

Goran Goransson

Received 5 August 1964

Abstract

Goransson G *The metabolism of fatty acids in the rat VI Arachidonic acid* Acta physiol scand 1965 *64* 1—5 — H labeled arachidonic acid and C labeled palmitic acid were simultaneously injected into male rats Arachidonic acid was preferentially incorporated into liver phospholipids as might be expected from the preponderance of this fatty acid in liver phospholipids as compared to glycerides Initially the labeled arachidonic acid and the labeled palmitic acid were oxidized at similar rates but at later times more arachidonic acid than palmitic acid was recovered from the whole animal This may be explained by the preferential incorporation of arachidonic acid into phospholipid in combination with the low turnover rate of the phospholipids especially those containing essential fatty acids The pattern of label in the blood glycerides closely resembled that in the corresponding liver fraction

The metabolism of several common non essential fatty acids (palmitic oleic stearic and palmitoleic) has been studied in our laboratory (Goransson and Olivecrona 1964 1965 Goransson 1965 a b) Experiments with a more rare non essential fatty acid (arachidic acid) have also been performed (Goransson 1965 c)

The present paper reports the results from experiments in which an essential fatty acid (arachidonic acid) and palmitic acid were simultaneously injected intravenously into male rats in different nutritional states Tissue distribution oxidation and recirculation of radioactivity into the blood were studied

Methods

The rats the method of preparing the injection solution the operative procedures and the analyses performed on the samples have been described earlier (Goransson and Olivecrona 1964 1965) Special care was taken in the present work to avoid auto-oxidation of the poly unsaturated fatty acids in the samples

The average recovery of 89 of the labeled arachidonic acid from the SiO columns as compared with the recovery of 92 % of the labeled palmitic acid was taken as evidence that the arachidonic acid was not oxidized during the analyses

The 5 6 8 9 11 12 14 15 H arachidonic acid (specific activity 721 mC/mmole) was courteously provided by F Hoffman La Roche & Co Basel According to their analyses *the arachidonic acid contained 4 % of by products mostly with a chainlength of 20 carbon* atoms but with differing number and location of double bonds No attempt was made to purify the arachidonic acid

The 1 C^{14} palmitic acid (Batch 22 Specific activity 3 75 mC/mmole) was delivered by The Radiochemical Centre Amersham England It was purified as described earlier (Goransson and Olivecrona 1964)

The injected dose of serum (0 5 ml) contained approximately 0 01 μeq of labeled arachidonic acid and 0 1 μeq of labeled palmitic acid

Results

The results have been expressed as the ratio H^3/C^{14} radioactivity in the separate lipid fractions The ratio in the injected fatty acids was taken as 1 0

Tissue distribution of radioactivity The distribution of radioactivity over liver adipose tissue muscle tissue heart kidneys lungs and spleen 5 and 320 min after the intravenous injection is shown in Table I

The ratio H^3/C^{14} radioactivity in the fasted rats at 5 min was greater than unity in some organs and less in others In the liver neutral lipids there was less H^3 than C^{14} radioactivity whereas the reverse was true for the liver phospholipids

In the fasted rats the H^3/C^{14} ratio rose during the interval of 5 to 320 min in all the organs studied In the whole rat the ratio equalled 1 0 at 5 min and 1 3 at 320 min

TABLE I Ratio H^3/C radioactivity in tissue lipids from rats after the i v injection of H arachidonic and C *palmitic acid in rat serum The ratio in the injected fatty acids* was taken as 1 0

Nutritional state	Minutes	Liver			Adipose tissue	Muscle tissue	Heart	Kidneys	Lungs	Spleen	Carcass	Whole rat
		Total	NL	PL	Total							
Fasted	5	1 1	0 7	1 6	0 8	0 9	1 7	1 3	1 6	1 1	0 8	1 0
		1 0	0 7	1 9	0 8	0 8	1 5	1 4	1 5	1 0	0 8	1 0
Fasted	320	1 7	1 1	1 8	0 9	1 1	5 9	1 9	1 9	2 4	1 1	1 4
		2 1	1 2	2 1	0 8	1 1	5 1	1 9	1 8	2 6	1 1	1 3
Refed	5	1 3	0 5	1 4	0 7	0 6	0 5	1 3	1 1	0 7	0 9	1 0
		1 3	0 6	1 4	0 6	0 8	0 5	1 2	1 0	0 8	0 9	1 1
Refed	320	1 3	0 5	1 3	0 5	1 0	0 2	1 3	1 2	1 0	1 1	1 0
		1 5	0 6	1 4	0 4	0 7	0 1	1 5	1 4	1 8	0 8	1 0

TABLE II Ratio H^3/C^{14} radioactivity in blood lipids in rats after the i v injection of H^3 arachidonic and C^{14} palmitic acid in rat serum The ratio in the injected fatty acids was taken as 1 0

	Fasted rats			Refed rats		
Min	Cholesterol esters	Glycerides	Phospholipids	Cholesterol esters	Glycerides	Phospholipids
20	—	1 7	—	—	0 9	—
	—	1 8	—	—	1 2	—
40	1 4	0 8	3 2	2 2	1 0	2 0
	2 0	1 0	3 5	1 6	0 9	2 3
80	3 5	1 1	2 5	2 8	0 8	1 4
	3 8	1 1	2 6	—	1 5	1 6
160	5 2	2 1	2 2	5 2	1 1	2 0
	5 9	2 6	3 3	4 3	1 7	1 8
320	10 4	—	2 8	4 4	—	1 5
	9 0	—	2 9	4 2	—	2 2

In both the refed and fasted animals the H^3/C^{14} radioactivity ratio was in some organs greater and in others less than unity Similar values were found at 320 min as at 5 min in all the organs as well as in the liver neutral lipids and phospholipids in the refed rats

The only major difference between fasted and refed rats at 5 min was found in the heart where the ratio in the fasted rats was considerably higher than that in the refed rats At 320 min the ratio was consistently higher in the fasted rats

Recirculation of radioactivity back into the blood The H^3/C^{14} radioactivity ratios in the blood lipids from 20 to 320 min after the injection are showed in Table II In both nutritional states the ratio in the cholesterol esters rose throughout the time period studied At 40 min the ratio in the glycerides had a value close to unity while the phospholipids displayed a ratio well above unity from 40 min onwards

Discussion

In the injection solution used in this work only 96 % of the injected H^3 radioactivity was present as arachidonic acid This fact would make the interpretation of curves representing the disappearance of radioactivity from the blood difficult and accordingly no attempt was made to obtain such curves However the amount of impurity could not significantly affect the values for tissue distribution and recirculation of radioactivity back into the blood

Uptake from the blood and esterification Both arachidonic and palmitic acid were rapidly extracted from the blood by all the organs studied In the refed rats where virtually no oxidation takes place during the first few minutes after injection (Goransson and Olivecrona 1964) *the values at 5 min suggest that the separate organs extract*

the two acids at different rates assuming that no transport of labeled fatty acids takes place between separate organs during the first 5 min after injection

Tissues rich in glycerides such as adipose tissue and muscle tissue contained less arachidonic acid label than palmitic acid label This is in agreement with the finding that arachidonic acid is preferentially incorporated into phospholipids in the liver

The low ratio of arachidonic acid label to palmitic acid label in the heart may result from a preferential uptake of palmitic acid as it has been shown that the heart extract separate fatty acids at different rate (Carlsten *et al* 1963)

Oxidation The fasted rats provide means of comparing the oxidation of the labeled arachidonic and the labeled palmitic acid

During the first 5 min the 2 acids were oxidized at the same rate This suggests that immediately after the entry of the 2 fatty acids into the cells they are both equally available for oxidation After 320 min more arachidonic acid label than palmitic acid label was recovered from the whole animal as well as from the individual organs and lipid fractions This may depend on the observed preferential incorporation of arachidonic acid label into phospholipid and the slow turnover of this lipid fraction (Dittmer and Hanahan 1959 Goransson and Olivecrona 1965) In addition phospholipids containing essential fatty acids have been proposed by Morin and Alfin Slater (1964) to have a lower turnover rate than other phospholipids

The suggestion that the slower oxidation of labeled arachidonic acid at later times is due to the preferential incorporation of this fatty acid into phospholipids is supported also by the finding that in organs rich in glycerides (adipose tissue and muscle tissue) the ratio of arachidonic acid label to palmitic acid label does not increase with time to the same extent as in other organs

Recirculation of radioactivity back into the blood Measurable amounts of radioactivity appeared in the blood cholesterol esters 40 min after the injection of the free fatty acids The ratio was greater than unity at that time and it rose to approximately 4 in the refed rats and to 10 in the fasted animals at 320 min This is not unexpected in view of the high percentage (55 %) of arachidonic acid in the plasma cholesterol esters found by Goransson and Olivecrona (1964)

The blood glyceride radioactivity rose to a maximum at 40 min after the injection At this time the ratio in the blood glycerides was similar to that in the liver neutral lipids at 5 min These findings are taken as additional evidence that blood glycerides originate mainly in the liver (Laurell 1959)

The blood phospholipid radioactivity showed a maximum at 160—320 min At this time the ratio between arachidonic acid label and palmitic acid label in the blood phospholipids and the ratio in the same fraction in the liver were not quite similar However the difference was not sufficient to make the present data incompatible with earlier results showing that the blood phospholipids originate in the liver (Fishler *et al* 1943)

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From the Institute of Neurobiology Medical Faculty University of Goteborg Sweden

The Steady State and Endogenous Respiration in Neuron and Glia

By

Holger Hydén and Paul W Lange

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Abstract

Hydén H and Lange P W *The steady state and endogenous respiration in neuron and glia* Acta physiol scand 1965 64 6—14 — The oxygen consumption of individual surviving nerve cells and glial cells was studied for two hours periods An extrapolation procedure was worked out which permitted the determination of the oxygen consumption rate in the steady state *in vivo* and the substrate amount for endogenous respiration The oxygen consumption rate of the neurons exceeded that of the glia by at least ten times in the steady state The values obtained were used for calculation of the amount of glucose per nerve cell which was found to be 5 per cent of the dry weight of the cell corresponding to a 70 mM glucose solution The amount of glucose in glia is 5 to 10 times lower than that of nerve cells Addition of glucose or β hydroxybutyrate to nerve cells having respired without exogenous substrate for two hours highly increased the respiration rate In neurons with increased RNA synthesis and respiratory enzyme activities the respiration rate and substrate level were low

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The following study deals with the steady state and the endogenous respiration in nerve cells and the surrounding glia Endogenous respiration has been defined as follows the respiration occurring in cells under conditions where no exogenous substrate is added (Lamanna 1963 *There has been pointed out however that a definition is difficult since the metabolism of most exogenously provided substrates is an endogenous* process A study of endogenous respiration in mammalian cells is further complicated by the regulatory mechanisms existing between the metabolism of exogenous and endogenous substrates The aim with the present study is to add further information on the metabolic behaviour of neurons in comparison to that of the surrounding glia since a regulatory mechanism has been shown to exist between these two types of cells from a biochemical as well as from a functional point of view (Hydén and Lange 1962 Pomrato and Hydén 1963

Preliminary observations during a kinetic study of enzyme activities in brain cells made it seem probable that the substrate for endogenous respiration differed in nerve

cells compared with the surrounding glia (Hydén and Lange 1962) In previous studies it was found that respiratory enzyme activities are higher in the glia than in the neurons which they surround (Hydén 1959) Inverse enzyme changes and RNA and protein changes occurred however in these cells during increased neural function (Hydén and Pigon 1960) A pertinent question is therefore if substrates for endogenous respiration also reflect functional changes

In the present study an extrapolation procedure is given by which the rate of endogenous respiration and the amount of its substrate can be estimated It will be shown that the substrate level for endogenous respiration is low in the glia and considerable in the neurons the magnitude is around 1 relative unit in the glia compared to that of 10 units in the neuron The endogenous substrate diminishes to a level close to zero in the latter at periods of increased RNA and protein synthesis with high respiratory enzyme activities

Material and experimental set up

One hundred and seventy white rabbits weighing 1 5—1 6 kg were used and 3 to 4 Deiters nerve cells or glia samples were taken from the lateral vestibular nucleus of each animal The animals were killed by an intravenous air embolus which rendered the rabbit unconscious within a few seconds after which the carotides were severed The skull was rapidly opened and a thick section through the lateral vestibular nucleus was placed in a substrate solution This phase of the procedure took 3 minutes

The Deiters nerve cells or the surrounding glia — the dry weight of each of which was 2 10 g — were isolated from the fresh section by free hand dissection at 80× magnification The cells were dissected and handled for the manometric determination according to one of the following procedures (a) The cell specimens were dissected from the section at room temperature and immersed in a standard medium of 37 C The temperature of the medium was kept constant by a thermocouple inserted in the medium and connected to an infrared radiator (b) The samples were removed at 37 C the air temperature being kept constant by a regulated infrared radiator placed at 0 5 m from the stereo-microscope (c) The samples were dissected and handled in the medium at + 4 C For this purpose a special microscope table was constructed It consists of two metal plates between which a freon liquid is circulating from a 1 horse power freezing unit provided with an additional air cooler and temperature regulator

The time interval between killing of the animal and placing the cell samples in microdivers for subsequent manometric measurement was 8 to 10 min The three microdivers each containing one or two nerve cells or one glia sample were then introduced into the thermostate bath (37 ± 0 005 C) (Hamberger 1963) at approximately five minutes after dissection of the cells In each experiment one microdiver contained the standard medium but no biological sample and served as a blank

The isolation of nerve cells with disruption of their axons and the finer terminals of the dendrites seems to be a drastic measure which could be expected to irreversibly damage the cells Yet such isolated cells together with clusters of surrounding glia have been cultivated in suitable media (Hansson and Sourander 1964) Deiters nerve cells and their glia have been cultivated at this laboratory for periods of months Thus these cells constitute an *in vivo* material A pertinent question is whether such isolated nerve cells will undergo morphological changes in the course of our experiments

In the phase contrast microscope no morphological changes could be detected in the cells at the end of the experiment four hours after the removal of the cells Hamberger (1963) found no structural changes of such nerve cells and glia which had remained in microdivers for 24 hrs

Determination of oxygen consumption per nerve cell or glia cell sample was carried out using a modified microdiver technique (Hydén and Pigon 1960) according to Zeuthen (1953) The volume of the divers was 0 2 to 0 4 µl Not more than one nerve cell or glia sample was placed in each diver Four divers were run at each experiment, one of which was a blank without biological sample Each cell sample as mentioned earlier had an average dry weight of

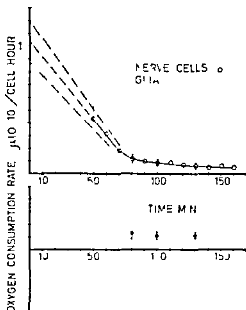


Fig 1 The oxygen consumption rate of nerve cells and glia expressed as µl O_2 10 ⁴/sample as a function of time after removal of the cells from the living animal The standard deviation of the means (standard error) is given for the first four sets of values used for the extrapolation and for two further sets of values measured at the decreased oxygen consumption rate The averages of the first four sets of values for the nerve cells are significantly different from the zero (blank) level with a high degree of significance contrary to those of the glia *cells which are not significantly different* from zero

20 000 µ/g (Hydén 1959) All solutions used in these experiments were preconditioned by air bubbling for several hours

The standard medium used was the following
Mg SO 6 7 mM
KCl 25 mM
Na phosphate (pH 7 4) 13 3 mM
Cytochrome C (Sigma) 0 02 mM
Sucrose 0 25 M

Extrapolation procedure

The aim with the present procedure is to utilize the first rate measurements of the oxygen consumption for extrapolation back to the time of removal of the brain cells

In all experiments a reference diver without biological sample was used as a blank Random variations of the behaviour of the divers with sample and the reference diver influence the values of the measurements of a single series of microdivers The behaviour of the divers during this first period was therefore averaged and calculated statistically in the following way

The rate of the change of the manometer recordings were noted at ten minutes intervals for every diver during two hours Thus in the present study 69 series of measurements on divers containing nerve cells from 45 animals and 27 series of measurements on divers containing glia cells from 20 animals were performed together with 42 simultaneous series of measurements on blank divers Thus for every ten minutes there is a series of 69 rate values for divers containing a nerve cell 27 rate values for divers containing glia cells and 42 rate values for the reference diver The average and standard deviation of the data from these three series are calculated The difference between the average rate value for the nerve cell divers and that of the reference divers gives an average value of the oxygen consumption rate of the nerve cells In the same way an average value of the oxygen consumption rate for glia cells was calculated These average values are recorded in Fig 1 together with their standard deviation in which

TABLE I Standard error of the mean of divers containing nerve cells (S_d) glia cells (S_{gd}) and of reference divers (S) The standard error of the oxygen consumption rate for nerve cells (S) and glia cells (S_g) are given by $S = \sqrt{S_d + S}$ and $S_g = \sqrt{S_{gd} + S^2}$

Time min	S_d	n	S_d	n	S	n	S	S_g
50	0 5	58	1 6	25	0 9	39	1 0	1 8
60	0 4	6?	0 9	26	0 5	40	0 6	1 0
70	0 3	69	0 9	27	0 4	42	0 5	1 0
80	0 2	69	0 4	27	0 3	42	0 4	0 5
100	0 1	69	0 2	27	0 2	42	0 2	0 3

n = number of measurements

are thus included both the statistical spread of the divers contain ng the biological sample *and* the statistical spread of the behaviour of the reference divers (see further Table 1)

The curve showing the oxygen consumption rate of the nerve cells is extrapolated back to the time of the removal of the cells from the living material This extrapolated value should give the oxygen consumption rate of the nerve cells in the steady state *in vivo* "The standard deviat on of this extrapolated value is not calculated with the a d of statistics but is also obtained as a result of extrapolat on of the standard deviation of the means on the curve as indicated in F g 1

Comments on the method

The following observations support and make the view conclus ve that the rate measurements presented in this study can be used as measure of the rate of endogenous respiration and that the area under the curves represents the amount of substrate available in each cell for endogenous respiration

First nerve cells were placed in the standard medium without substrate for 2 hrs Then the cells were transferred to divers and the oxygen onsumption measured The values were found to lie close to the ero level as denoted by the dotted line close to zero in Fig 2 This is a con clusive information that the initially high respiration rate is a function of endogenous substrates only found in fresh cells and do not represent techn cal errors

Secondly the addition of glucose and β hydroxybutyrate to nerve cells and glia after two hours in a medium without substrate st mulated the oxygen consumption and raised the curves from a zero level to average values between 5 and 10 / 1 O 10 per cell sample and hour See below Fig 2

Thirdly in the experiments w th induced RNA synthesis by tricyano-amino-propene the amount of RNA and proteins in μμg cell increased by 25 and the cytochrome oxidase and succinoxidase activities measured as μl O 10 per cell and hour rose by 300 per cent In spite of this fact the resp ration rate curves of such nerve cells lie close to the zero level See Fig 3

Fourth when rabbits injected with tricyano-amino-propene were placed in an tmosphere with decreased oxygen tension (5 O 95 N) they d ed ea lier compared to the control rabb ts subjected to the same oxygen tens on. These findings which w ll be presented below (see p 6) const tute further support for the conclusion that the area under the extrapolated respiration rat curves presented in this study represent a measure of the substrate level for endogenous respiration in nerve cells and in glia

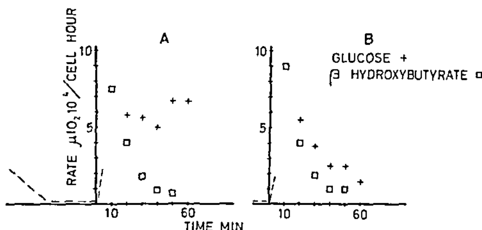


Fig 2 The effect of the addition (at zero time) of glucose and β hydroxybutyrate to nerve cells (A) and glia (B) having respired for 2 hrs in standard medium without substrate

Results

Control materi l The results from analyses of 69 nerve cells (45 animals) respiring in the standard medium are summarized in the curves of Fig 1 and in the same figure are presented the results on the glia a total of 27 analyses from 20 animals as described on p 3

The oxygen consumption rate of the glial cells is 0 to 1 μl O_2 10 per cell and hour By contrast the first rate measurements of the oxygen consumption of the nerve cells deviate from the zero value with a high degree of significance as can easily be cal culated by applying conventional statistical tests These values can therefore be used for extrapolation back to the time of cell removal as demonstrated in Fig 1 The extra polated value thus obtained for the nerve cells lies around 10 μl O_2 10 ⁴ per cell and hour This means that the oxygen consumption rate of nerve cells *in vivo* of these control animals exceeds that of the glia by at least ten times

The data obtained can also be used for a calculation of the amount of substrate for endogenous respiration in the neurons The respiratory quotient of the brain lies close to 1 0 which means that the brain tissue covers its energy demands mainly by glucose (Kety 1962 p 113 There is no reason to believe that the Deiters nerve cells and glia differ in R Q value from that of the average brain Furthermore Gainer Allweis and Chaikoff (1963 have found that in intact animals all metabolic CO_2 is derived from glucose

In this study we found the oxygen consumption rate per nerve cell at zero time (*i e in vivo*) to be 10 10 l O_2 per cell and hour Based on the figure 2 10 g as the dry weight of these neurons (Hydén and Pigon 1960) the oxygen consumption can be recalculated as 50 ml O_2 per g dry cell weight and hour In order to calculate the glucose content available for endogenous respiration the area under the oxygen consumption rate curve was calculated It was assumed that the oxygen consumption per cell was zero after 1 5 hour which is a reasonable valu judged from Fig 1 During this time 50 ml O per g per hour 1 5 hour 1 2 is consumed *i e* 37 5 ml O_2 per g of dry weight

This means using R Q = 1 $\frac{37.5 \cdot 10^{-9}}{6 \cdot 22.4}$ mol glucose = 50 mg glucose/g dry weight available for endogenous respiration

Thus 5 per cent of the dry weight of the nerve cell consists of glucose and that means for the Deiters nerve cells an amount of 1 000 *µµg* of glucose The amount of glucose in the same dry weight of glia is then more than 10 times less Considering the regulative mechanisms of the cell with respect to intermediary metabolism these calculated values must be considered as maximal glucose values (Danforth and Wilson 1961 Geiger Kawakita and Barkulis 1960) The glucose dissolved in the nerve cell will correspond to a 70 mM glucose solution This value is high but not unreasonable Geiger (1962 p 128) reported values of 50 mg glucose per 20 g of dry cortex of cats Taking the glia to neuron ratio as 3 (cf also Hess) this amounts to a glucose content of 10 mg per g dry nerve cell neglecting the glucose content of the glia

Addition of glucose and β hydroxybutyrate to nerve cells and glia having respired in standard medium for two hour

The oxygen consumption rate of the nerve cells was found to be close to the zero level after two hours of respiration in the standard medium Fig 2 (A) demonstrates that the addition of 12 5 mM glucose or 10 mM *β* hydroxybutyrate increased the respiration rate in a striking way In all 20 glucose and 15 hydroxybutyrate experiments were performed on altogether 25 animals

Fig 2 (B) shows results from similar experiments on the glia from altogether 18 animals divided on 14 glucose and 11 hydroxybutyrate experiments

As is seen from the curves in Fig 2 the addition of these substrates raised the respiration rate to high values

Thus the increased respiration rate values made possible by the addition of exogenous substrates demonstrate that the cells are functioning and furthermore that the decreasing rates in the control experiments reflect endogenous reactions of nerve cells and glia Using isolated Deiters nerve cells in a Krebs Ringer solution and tritium labeled cytidine it could be shown by microelectrophoresis that such nerve cells incorporate cytidine into RNA which is synthesized (Brattgård and Hydén)

Endogenous respiration of nerve cells during induced RNA synthesis

The administration of tricyano-amino-propene (20 mg/kg body weight) increased the amount of RNA per Deiters nerve cell by 25 from an average of 1550 *µµg* to 2100 *µµg* of RNA per cell in one hour (Egyhazi and Hydén 1961) The base ratio composition of this synthesized nuclear and cytoplasmic RNA proved to be changed with respect to the guanine and cytosine values (Hydén and Egyhazi 1962) As was pointed out above the respiratory enzyme activities were likewise increased

Fig 3 shows that the oxygen consumption rate of nerve cells with increased RNA content respiring in standard medium does not significantly differ from the zero level Nineteen nerve cells from 13 rabbits were used

Twelve analyses on glia from 10 rabbits having received tricyano-amino-propene were carried out All rate curves obtained lie close to zero and did not significantly differ from those of control animals

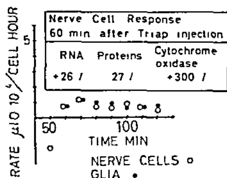


Fig 3 The oxygen consumption rate of nerve cells and glia cells of the animals after tricyano-amino-propene treatment Zero time 1 hr after the i v administration of tricyano-amino-propene

It has been found that a moderate hypoxia of 8 % O_2 and 92 % N_2 for 15 hrs in rabbits increased the cytochrome oxidase activity and the anaerobic glycolysis significantly of the Deiters nerve cells (Hamberger and Hydén 1963) The effect of a more severe hypoxia of 3 % O and 97 % N on the survival times of rabbits after administration of tricyano-amino-propene was therefore studied Seven control and 13 experimental rabbits were used A glass cage with a flow rate of 3 lit per minute was used (Hamberger and Hydén 1963) The CO_2 content of the gas used did not exceed 0 35 % The animals in which increased RNA and protein synthesis of nerve cells occurred did only survive for half the time during which the control rabbits could stand the decreased oxygen tension A decreased resistance towards hypoxia was thus observed in the animals having nerve cells with oxygen consumption rate curves close to the zero level This could be expected if the demands of synthetic activities had lowered the substrate of the cells available for endogenous respiration Thus if the oxygen consumption rate of the control nerve cells in standard medium would reflect simply enzyme activities then this oxygen consumption rate should be observed even during RNA and protein synthesis or it should be above normal But the rate curves of these nerve cells lie close to zero Since such intense synthesis of polymers requires energy it is clear that the oxygen consumption rate curves presented in this study do not simply reflect enzyme activities but with a high probability demonstrate a change in the level of substrates available for endogenous respiration

Discussion

In the present study special efforts have been made to stabilize the experimental procedure used for the measurements of oxygen consumption rates even during the first hour after the microdivers used were introduced in the thermostate bath An average blank value (divers without cells) obtained from a great number of measurements under standardized conditions was used Measurements of oxygen consumption rate could then be performed 40 minutes after the removal of the brain cells and 5 to 10 minutes after inserting the divers in the thermostate bath

The extrapolation procedure used in the study was thus based on a great number of measurements taking the difference between the values of nerve cells and glia and that of the reference divers Therefore from a methodological point of view the basis of the study was well established On the other hand the authors are well aware of the

caution needed in applying an extrapolation procedure with due respect to its inherent weakness in elucidating a time gap. The consistent results and the plausibility of the quantitative data obtained support methods and conclusions about the *in vivo* conditions of the cells.

It is obvious that the oxygen consumption rate of the nerve cells exceeds that of the glia by at least ten times. Previous analyses of isolated Deiters' nerve and glial cells have shown that the cytochrome oxidase and succinoxidase activities are highest in the glia but can reverse during increased functional demands (Hydén and Pigon 1960). The present results constitute no contradiction since the activities of single enzyme systems are no measure of the total integrated process, in this case the oxygen consumption. Our data that the oxygen consumption of the whole brain mainly reflects that of the neurons agree with those of Hess (1961, p. 200).

The experiments with exogenous addition of glucose and β-hydroxybutyrate and measurement of rate curves during increased RNA and protein synthesis show according to our view that the oxygen consumption rate curves reflect the endogenous respiration and that the integral of the curves constitute a measure of the amount of substrates available in the cells. The maximal amount of glucose, 1 000 $\mu\mu$g calculated per nerve cell, i.e. about 5 per cent of the total cell body weight of 20 000 $\mu\mu$g seems plausible.

The neurons are capable of synthesizing RNA and proteins as part of a functional response. When the synthesis of such polymers is high as in our experiments with tricyano-amino-propene, it is interesting to note that the level of endogenous substrates decreases. It agrees with known facts about the need of energy for polymer synthesis. It further stresses the need for more knowledge about the action of psychopharmaca to which the tricyano-amino-propene belongs.

The results presented in this study indicate a rapid replenishment of substrates directly to the neurons at increased functional demands.

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Some Mechanical Aspects of an Intestinal Smooth Muscle

By

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Abstract

Åberg A K G and J Axelsson *Some mechanical aspects of an intestinal smooth muscle* Acta physiol scand 1965 *64* 15—27 — Atonic pieces of taenia coli muscle were obtained with the aid of adrenaline which abolishes spontaneous spike discharge As reference length the in vitro length at 37 C relaxed with adrenaline was used L_o (isometric) is arbitrarily defined as the greatest length of an inactive muscle when the force applied to straigthen it does not exceed 50 dynes At this length the muscles were found to develop *maximum* tension L_o (isotonic) is the pre loaded length of a relaxed muscle These *conditions* gave results which were repeatable with reasonable statistical accuracy other conditions gave less reproducible results Force velocity curves obeying Hill's equation $(P+a)(v+b) = (P_o + a) b$ were obtained Maximum velocity of shortening of an unloaded muscle was extrapolated to 30 per cent of Lo/sec Velocity of shortening appeared related to the ratio P_o P Rate of tension development was related to muscle length and resting tension with a maximum at 170 to 180 per cent of L_o The highest rate measured was [illegible] 800 dynes/sec Maximum physical work was performed with constant load of about 30 per cent of P_o. Maximum shortening of the muscles under 100 dynes tension varied from 60—80 % The results are discussed in relation to other types of muscle

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The experimental work presented here was performed on the taenia coli of the guinea pig The purpose of this work was to try to establish standardized experimental conditions permitting the collection of mechanical data and a more quantitative treatment of some earlier findings (Axelsson and Bulbring 1961 Axelsson 1961)

The paper describes the relationships between (a) external force and velocity of shortening (b) muscle length and rate of isometric tension development (c) maximum isometric tension at L_o (for definition see Methods) and velocity of shortening (d) tension and physical work (e) length and tension (resting and active) and other mechanical properties

Methods

Male guinea pigs weighing 250 to 350 g were used They were stunned and bled out

Dissection and pretreatment Pieces of taenia coli were dissected in conditions which are known to abolish spontaneous spike discharge This was achieved (1) by cooling and (2) by treatment with adrenaline (Bulbring 1954 1957 Burnstock 1958) (1) At temperature below 15 C the muscles fail to exhibit spontaneous discharge of spikes. During dissection the whole muscle and underlying colon were therefore immersed in modified Krebs solution The temperature of the solution was 10 C it rose to about 12 C during dissection

(2) To obtain "inactive *in situ* length at 37 C the whole muscle was immersed in Krebs solution containing adrenaline in the concentration 2 × 10 w/

The length of the muscles before dissection in either of the above described conditions will be referred to as inactive *in situ* length

After being carefully dissected free from connective tissue the pieces were transferred to a small dish containing the same kind of solution and kept at the same temperature Hooks of stainless steel (each weighing 10 mg) were tied on to each end

The muscles were then mounted in the recording apparatus still in the same solution and at the same temperature and their "inactive *in vitro* length was determined

For experiments done with isometric recording inactive *in vitro* length is arbitrarily defined as the greatest length of an "inactive muscle when the force applied to straighten it does not exceed 50 dynes The external force applied to determine "inactive *in vitro* length was constant for each muscle The mean *in situ* diameter of the muscles was 1 2 ± 0 2 mm The variations in diameter do not affect the validity of this operational definition for practical purposes

For experiments done with isotonic recording the definition is modified by stating the pre loaded length of an "inactive muscle under loads varying from 100 to 12 000 dynes

After the first determination of "inactive *in vitro* length the muscles were except otherwise stated allowed to recover for 60 min at 37 C in normal solution and their "inactive length was then again determined before beginning the experiment

Solutions

Normal solution The solution referred to as normal solution was a modified Krebs solution Its composition was as follows (mM) Na 137 47 Ca 2 49 K 5 93 Mg 1 19 Cl 131 11 HCO 15 48 H PO 1 19 glucose 11 5 It was aerated with a mixture of O (97) and CO (3) Its pH was 7 25

High potassium solution This solution contained 128 mM K and was obtained by replacing the NaCl of the normal solution with KCl On a few occasions the NaHCO of the normal solution was replaced with KHCO The solution then contained 143 40 mM K *i e* about 24 × the normal concentration

Stimulation Three types of stimulation were used electrical stimulation by square waves of varying amplitude and duration drugs of varying concentrations and general reduction in membrane potential by raising the external potassium concentration

Electrical stimulation by square waves The device for electrical stimulation was similar to that described by Burnstock (1958) It was used in experiments where the mechanical response was measured isometrically simultaneously with registration of the electrical activity

Chemical stimulation

1 *Drugs* The drugs used for stimulation were acetylcholine and carbachol The lowest concentrations of each drug which gave a maximum mechanical response in normal solution at 37 C were determined Twice the concentration at which the mechanical response was not further increased is referred to as supramaximal stimulus The following concentrations were used acetylcholine chloride ? 10 w/v carbachol chloride 2 × 10 w/v

2 *High external K* We obtained a maximum mechanical response with 118 mM K The supramaximal stimulus used under both isometric and isotonic conditions was 128 mM K

Mechanical recording technique

Isotonic measurement Two types of transducers were used (1) differential transformer (2) mechano-photoelectric transducer

In the construction of the transducers, care was taken to reduce forces modifying the records of the muscle response

The output from both transducers was recorded on a Grass polygraph model 5 D

Isometric measurement The force displacement transducers used for isometric recording of muscle tension were of 2 different manufactures Grass type FT 03 and RCA 5734 The displacement per unit load was FT 03 1 μ/g and RCA 5734 3 μ/g The output potential was recorded either directly on the ink writer or used for displacement of one beam of an oscilloscope and recorded photographically

Combined measurements The apparatus described below enabled both isotonic and isometric measurements to be made in one experiment without disconnecting the muscle

The apparatus consisted of 2 functionally independent parts Isometric measurements were done with the same type of transducers as described above A plastic coated wire connecting muscle and isometric transducers consisted of 2 parts the free endpoints of which were rigidly held together with the aid of a small electromagnet Immediate change from isometric to isotonic measurements was possible by interrupting the current activating the magnet The isotonic measurements were done with a photoelectric transducer

Recording of electrical activity For simultaneous recording of electrical and isometric mechanical activity we used the sucrose gap method (Stämpfli 1954)

The application of this method to smooth muscle was described by Burnstock and Straub (1958)

Results

A In situ length of an inactive muscle

The *in situ* length of an inactive striated muscle has been used as reference length and called L_0 (Wilkie 1954) At about that length striated muscle has been found to develop maximal tension in response to tetanic stimulation Ramsey (1960) defines L_0 as the length at which the muscle develops maximal tension

Taenia coli on the other hand when not stimulated is neither atonic nor inactive At body temperature it continuously changes its mechanical state because of propagated potential changes spontaneously arising in the muscle itself

As described in methods inactive muscles were obtained either by cooling or by treatment with adrenaline We found however no consistency in the relationship between inactive *in situ* and inactive *in vitro* lengths The underlying colon is probably exerting force on the inactive muscle thereby determining its *in situ* length The inactive *in situ* length was therefore rejected as a basis for definition of L_0 for this muscle

B In vitro length of an inactive muscle

When the muscles were rendered inactive by cooling to 12 C and mounted isotonically at that temperature preloaded with 100 dynes constant load their length did not remain constant The method of cooling was therefore rejected as unsuitable for obtaining a reference length and the *in vitro* length in the presence of adrenaline at 37 C was investigated The following series of experiments was done to test whether that length remained constant while a given external force was applied

1 Isometric recording Pieces of muscle were dissected at 37 C in a solution containing adrenaline (see Methods) They were mounted isometrically still in the presence of adrenaline 50 dynes force was applied to straighten the muscles and their length under that force was determined The adrenaline was then washed out whereupon the muscles became spontaneously active and developed considerable tension Adrenaline was again applied after 30 min and the length of the inactive muscle under 50 dynes force re checked It was found that the relationship between external force and length of the adrenaline relaxed muscles remained constant for at least 4 hrs That length was called L (isometric)

If the muscles mounted in the condition described above were repeatedly subjected to supramaximal stimulation by high [K] carbachol etc (see Methods) a change in the ratio external force/muscle length was sometimes observed

2 Isotonic recording Adrenaline relaxed muscles were mounted isotonically under 100 dynes tension With 15 min interval the load was stepwise increased up to 10 000 dynes (When the load was changed a stopper saved the muscles from strecth) After the initial increase in length following increase in load a slow increase in deformation lasting for about 5 min was usually observed Retarded deformation of this kind occurring under constant stress has been called creep (for ref see Landowne and Stacy 1957) Thereafter the length became constant during the testing period of 15 min as long as no change in the experimental conditions was introduced On withdrawal or stepwise reduction in the external force the muscle length did not follow the same path as during increase in force This resulted in a formation of an hysteresis loop All the experiments described in this paper in which the muscles were subjected to external forces exceeding 50 dynes were done after the initial plastic changes had taken place and when the muscles followed identical paths upon application and withdrawal of the forcing agent

3 Metabolism and the inactive in vitro length Apart from the creep described above changes in the adrenaline relaxed length sometimes occurred after repeated supra maximal stimulation with constant external force The question arose whether these changes represented changes in tone in spite of no spike discharge or plastic changes in some passive elements of the muscle

Tone in taenia coli depends on metabolic energy supply for its maintenance There fore it was tested whether the inactive length could be affected by either withdrawal of glucose or by exposure to a metabolic inhibitor (mono-iodoacetate [IAA] concentration in the bath of 3×10^{-2} w/v)

In each experiment 4 muscles were mounted and their inactive lengths were determined in the presence of adrenaline as described above Two muscles were kept in normal solution as controls the other 2 exposed to glucose free solution or to IAA for 3 hrs Their inactive length was determined every 30 min No significant increase was observed in the inactive length of the 2 latter groups compared with the control groups

It is concluded that the adrenaline relaxed muscle at 37 C is atonic For both isometric and isotonic conditions the *in vitro* length of an adrenaline relaxed muscle is proposed as the most reasonable operational definition of L_0 for the purpose of our experiments When changes in that length occur during an experiment they are treated as changes in L_0

C Rate of contraction

In all experiments the muscles were relaxed by adrenaline before stimulation Supra maximal stimulation was carried out by applying a high $[K]_o$ in both isotonic and isometric conditions In addition in the isometric conditions acetylcholine and car bachol were used

1 Isotonic recording A condition for a strictly isotonic contraction is that the muscular force P is equal to the constant load F during the whole process of shortening Neglecting the frictional forces and the mass of the muscle (see Methods) the general equation for the shortenings described below can be written $I = I + Mv$ (M = effective mass $v = d^2x\,dt^2$ where x amount of shortening and t = time) To approach a true isotonic shortening where I I the term Mv must be made small compared to I

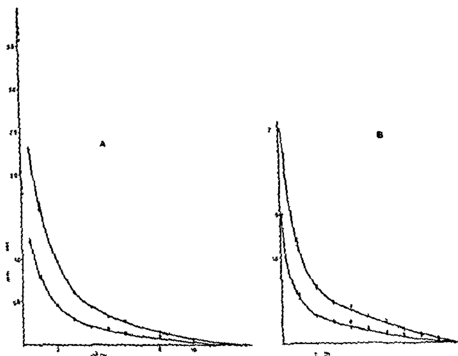


Fig 1 Force velocity curves. Abscissae force Ordinates velocity Upper curve maximum velocity of shortening Lower curve mean velocity of shortening At 100 dynes tension L_o was 19 mm (A) and 14 mm (B) respectively

For the experiments described here the force necessary to accelerate the whole system up to the maximum velocity was calculated to be 3 8 dynes during the initial acceleration (maximum velocity 3 7 mm/sec gained during 1 sec) Thus the forces due to inertia would be small compared with P at the velocities present in these experiments (P = maximum isometric force at L_0) However constant loads varying from 100—12 000 dynes were used Therefore during the acceleration from zero velocity to the velocity of a few mm per second Mv may in a few cases have become sufficiently great compared with F to cause a deviation from the condition of isotonic contraction

The purpose of these experiments however was to obtain a curve relating the velocity of shortening to isotonic force The maximum rate in each contraction was obtained from (a) the tangent at the point of inflexion or (b) by extrapolating the steepest linear part of the curve The values for mean velocities were obtained by dividing the total change in length by the time elapsed from the beginning of the shortening

A typical force velocity is shown in Fig 1 A. The curve fits Hill s equation $(P + a)(v + b) = (P_o + a) b$ = constant (Hill 1939) (P = the constant load P = the maximum isometric tension at L_0 v = dx/dt = velocity of shortening a and b constants with dimensions of force and velocity) Graphical determination of a and b according to the method of Katz (1939) showed that both were constant Determined for the maximum velocity of shortening against different afterloads of a 19 mm long muscle a and b were 800 dynes and 0 24 mm/sec respectively (Fig 1 A)

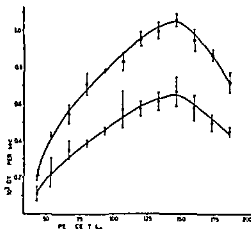


Fig. 2. The rate of tension development as function of muscle length. Abscissae: muscle length expressed as % of L_o. (L_o of this muscle was 7.5 mm). Ordinate: rate of tension development. Open circles: maximum rate. Closed circles: mean rate. The standard deviation at each length is shown in the figures.

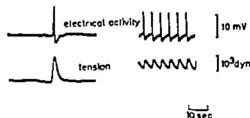


Fig. 3. Electrical and mechanical responses to electrical stimulation. For description see text.

Fig. 1 B shows a force velocity curve in which neither a nor b were constant. Such curves were obtained if the experiments were started before adrenaline had been given repeatedly for some time. It is believed that the changes in mechanical qualities occurring during the course of these experiments are secondary to a change in the metabolic status of the muscles.

The maximum velocity of an unloaded muscle was found to be approximately 30 per cent of L_o/sec (determined by extrapolating a number of force velocity curves). The maximum velocities measured at 100 dynes differed considerably from one muscle to another. The highest value which has been measured was 20 per cent of L_o/sec.

With increasing load the L_o of the preloaded muscles increased with a consequent decrease in cross-sectional area. Therefore the changes in stress (force per unit area) will be greater than the changes in tension (total force applied along the muscle to stretch it) which are indicated in the figure.

In a series of experiments the muscles were prevented by a stopper from increasing in length beyond the length of the resting muscles under the smallest tension. The velocity of shortening with increasing load of the after loaded muscles was not significantly different from that of the muscles which had been extended by increasing the load.

2. Isometric recording

a) Stimulation by high K

In Fig. 2 the maximum rate and the mean rate of tension development obtained at each length are plotted against the length expressed in % of L_o. In this muscle maximum

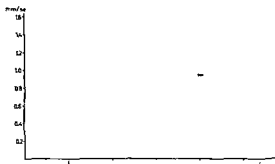


Fig 4 The relationship betwee 1 velocity of shortening and P Abscissae The tension in each contraction as fraction of the maximum tension developed by that muscle at L_0 Ordinate maximum velocity of shortening

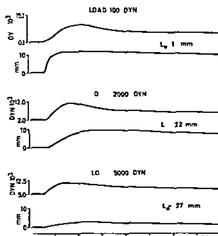


Fig 5 Isometric and isotonic contractions of the same muscle Upper trace isometric contrac tion Lower trace isotonic con tract on For description see text

rate was obtained at 145 , L In 12 muscles the length at which maximum rate was obtained varied from 131—180 per cent of L_0

As the rate of tension development appears to be a function of muscle length and thereby resting tension L_0 must be considered if the velocities of the contractile processes in isometric and isotonic conditions are to be compared The rate of tension development at L was influenced by the spontaneously maintained tension before the muscle was relaxed (The higher the tension the greater the rate)

The maximum rate recorded in these experiments was 2 800 dynes per sec

b) Stimulation by acetyl holine or carbachol

In a series of experiments various concentrations of acetylcholine and carbachol were used as stimuli Maximum response was obtained with acetylcholine at a concentration of 10 Carbachol 10 gave the same response as acetylcholine 10 and the maximum response with it was obtained at a concentration of 1 × 10 The difference in potency between the two drugs was eliminated by previous application to the muscles of a cholinesterase inhibitor (physostigmine concentration in the bath 1 × 10) The rate of tension development obtained with optimum concentration of each drug did not differ significantly from the rates obtained when high [K] was used as the stimulus

TABLE I Summation of the results obtained during isotonic and isometric contraction of one muscle under different resting tension

Resting tension 10³ dyn	Resting length % L_o	Maximum velocity of shortening mm/sec	Physical work 10³ erg	Maximum rate of tension development 10³ dynes sec	Tension developed 10³ dyn	Tension total (i e resting + tension developed) 10³ dyn
0 1	109	3 2	1 0	1 0	9 0	9 1
2 0	129	0 8	16 ?	1 25	9 0	11 0
5 0	159	0 2	10 1	1 0	7 5	12 5

c) Electrical stimulation

Fig 3 shows the tension response to electrical stimulation The forces developed in response to single stimuli are small compared with maximum isometric force (This is further considered on p 12) The maximum rate of tension development (V[max]) in response to single stimuli was 1 100 dynes/sec When the muscle was stimulated at higher frequency the tension never returned to the resting value between stimuli V (max) was then only 500 dynes/sec The recording was done with the muscle at about 110 % L_o

3 Isometric and isotonic recording from the same muscles Hill s equation (see p 5) rearranged into the form $V = (P_o - 1)\ b/(P + a)$ suggests that the velocity of shortening depends on the ratio between the maximum force which the muscle can develop and the force acting on the muscle Therefore in a series of experiments isometric force and velocity of shortening were measured in the same muscle The constant load F corresponds to Hill s P Fig 4 shows the results obtained from 5 muscles In all experiments the constant load was 2 000 dynes

Before and after each isotonic contraction the muscle was made to contract isometrically and the mean P was calculated As P_o may change during the course of an experiment different values for P_o/P were obtained from one and the same muscle The curve indicates that velocity increases with increasing P_o

In Fig 5 the isotonic and isometric contractions of one and the same muscle can be compared at 3 different lengths L_o in the isometric condition was 17 mm With 100 2 000 and 5 000 dynes resting tension the length was 109 % 129 % and 159 % L_o respectively under both preloaded isotonic and isometric conditions

The results obtained in this experiment are summarized in Table I

D The amount of isotonic shortening at various loads

The shortening of preloaded muscles under various loads when stimulated with high [K] are plotted in Fig 6 A and B The broken line is actual shortening in mm plotted against load The solid curve expresses the shortening as per cent of L_o at the given load

The initial increase in actual shortening (seen in the broken line in Fig 6 A) occurs because the muscle increases in length when the load is increased from 100 to 300 dynes This initial increase in length is relatively greater than the increase which occurs when

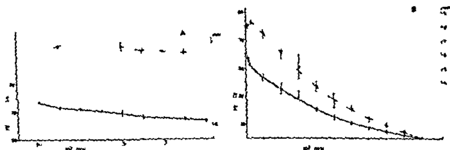


Fig 6 Isotonic shortening under various loads A Tensions of 100—900 dynes This shows in detail for one muscle what is indicated for another muscle at the beginning of B
B Another muscle loaded with tensions of 100—10 000 dynes Solid line shortenings expressed as % of the preloaded L_o For further description see text

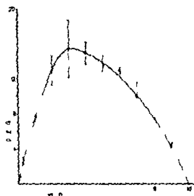


Fig 7 Physical work Abscissae load Ordinate work Vertical lines show standard deviations Each period is the mean of about 4 observations

greater loads are applied The shortening under 100 dynes load of the muscle shown in Fig 6 B was about 60 per cent of L_o

The shortening of 12 muscles under 100 dynes load expressed as percentage of L_o at the time of stimulation varied between 60 and 80 per cent

E Physical work at various loads

In Fig 7 the work done by a muscle at various loads is plotted It will be seen that maximum physical work is performed by this muscle under a load which is about 1 3 of the greatest load which the muscle can lift In ten muscles it was found that maximum work was performed with a constant load of 30 per cent of $P_o \pm 5$

The maximum physical work was found to be between $\frac{P \quad L_o}{7\ 0}$ and $\frac{P \quad L_o}{8\ 2}$

F Maximum isometric force

The maximum force developed by 19 muscles at L_o was $\frac{1\ 82 \pm 0\ 52\ \text{kg}}{\text{cm}}$ The area is the mean cross-sectional area of each muscle piece The values are obtained by making the assumption that $\frac{P_o \times \text{length}}{\text{volume}} \approx \frac{P_o}{\text{area}}$ There was however a poor correlation be

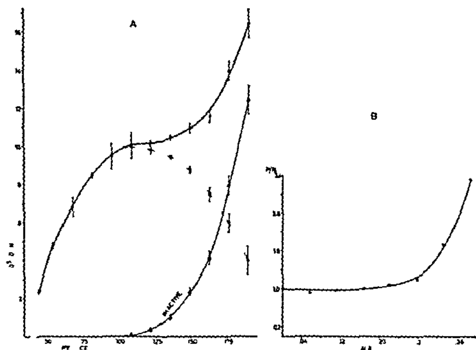


Fig 8 Length tension relationship For description see text.

tween length/volume ratios and the force developed by these 19 muscles This indicates that either the muscle pieces are not of uniform thickness or not homogeneous (i e not containing the same number contractile fibres per unit area)

The relationship between maximum force and the length of the muscle appears from Fig 8 A

G Length tension relationship

The curve labelled inactive (Fig 8 A) shows the length tension relationship in the resting muscle Adrenaline was given before each stretch The speed of lengthening was constant throughout the experiment Each point on that curve represents the stable tension maintained at that length When the tension was stable the muscle was stimulated by high [K] Each point on the upper curve shows the highest total tension (*i e* resting tension developed tension) during stimulation at that length The broken line shows the difference between the passive and active tension *i e* the tension developed in addition to the resting tension The maximum tension was developed by this muscle at about L_0 although the total tension continued to increase to at least 180 % L_0 This was a constant finding in all the muscles investigated

Fig 8 B shows the length tension curve of a depolarized (*i e* active) muscle The muscle was depolarized at L_0 by high [K] This produced a contracture with a maximum tension development of 1 200 dynes The tension which was maintained by the depolarized muscle was 8 000 dynes at L_0 In this state the muscle was stretched The ordinate

is the increase in the tension in terms of P i e P/P_o In this case P_o is the maintained tension and not the maximum On the abscissae the increase in length is plotted in terms of L The depolarized muscle could be stretched to about 130 per cent of L_o with increase in tension

Discussion

When first attempting to put the mechanics of the muscle on a quantitative basis we were faced with many difficulties Several authors working on different types of smooth muscle have discussed these difficulties among them Bulbring (1958) working on taenia coli and Lundholm and Mohme Lundholm (1962) working on mesenteric arteries These authors point out that as an isolated smooth muscle may maintain a varying degree of tone the change in response to stimulation may in many cases reflect only variations in this tone When stimulation ceases the tone may not return to its original level Spontaneous changes in muscle tone may occur during the experiment Under these conditions quantitative work on the mechanical properties of a muscle becomes extremely difficult The first task was therefore to obtain a reference length which one could return to and which represented an atonic state of the muscle

Reference length

We tried to abolish spontaneous spike discharge *in situ* before dissection in order to obtain more comparable muscle pieces However there was no consistent relationship between the adrenaline relaxed *in situ* length and the adrenaline relaxed *in vitro* length under constant external force We therefore rejected the inactive *in situ* length as a reference length According to our experience it is of limited meaning to talk about the natural length in the body as reference length for this muscle (Yoshida Toshiro 1963)

The adrenaline relaxed *in vitro* length at 37 C was used as reference length L_o (isometric) was arbitrarily defined as the greatest length of an inactive muscle when the force applied to straighten it is ≈ 50 dynes The length of the muscles under 50 dynes resting tension was only slightly greater than their initial length (the length of an inactive muscle when no external forces other than gravitational are applied) Application of greater force caused increase in muscle length with consequent decrease in diameter Length tension curves showed that maximum tension was developed at about L_o (Fig 8) Thus the L_o used in these experiments proved to be in good agreement with the definition of L_o given by Ramsey (1960) L_o (isotonic) denotes the resting lengths of a preloaded muscle In this condition creep and plastic changes occur causing change in the L_o at a given load We have avoided this problem by including only results obtained when the plastic changes had taken place (p 4)

We abandoned the idea of stretching muscles of different weights per unit length to the same numerical value for the weight/length ratio as we found that by doing so we were introducing very variable stress in the muscles without securing the same cross-sectional area (see p 10)

By our definition of L_o the different pieces of muscle will have the same resting tension and be kept at the length at which they develop maximum tension

In this condition (i e at L_o) we obtained results which were repeatable with reasonable statistical accuracy Other conditions proved to give less reproducible results

Velocity of shortening

We obtained force velocity curves in which values for a and b in Hill's equation were constant (Fig. 1 A). The deviations observed in some earlier experiments were supposed to be a consequence of stimulated metabolism and increased energy supply during the beginning of the experiments, which might have delayed the decrease in velocity as the load was increased. The decreased velocity of shortening observed with increasing load can be explained as a consequence of increased work and energy requirements per unit shortening. The findings that both velocity of shortening under constant load and the rate of tension development decreased when the external glucose was removed (see Axelsson, Högberg and Timms 1964) support the view that energy production may be a rate limiting factor in contraction (Fenn 1924, 1957, Hill 1938, Wilkie 1954).

Wilkie (1957) suggests that "the chemical reactions which produce muscular energy are themselves controlled by the force on the muscle". Table I shows that at 129 per cent of L_o the velocity of shortening was decreased 4 times compared with the velocity at 109 per cent of L_o. The work however increased 16 times and consequently the work per unit time increased with increasing force up to a certain limit. Similarly the tension developed per unit time increased with the force on the muscle up to a given limit for each muscle (Fig. 2). In experiments in which maximum isometric force and velocity of shortening were determined for the same muscles (Fig. 4) it was found that velocity increased with increasing P_o, as would be predicted from Hill's equation.

Figures for the velocity of shortening of taenia coli are much lower than those for striated muscle. Maximum velocity of an unloaded taenia was calculated as 30 per cent of L_o/sec (Fig. 1 A), whereas for an unloaded frog sartorius it can be calculated by extrapolation to 40—50 mm/sec or about 135 per cent of L_o/sec (Hill 1938, Wilkie 1957). Hill's values were obtained at 0° C. All our values were obtained at 37° C. Buchthal and Rosenfalck (1957) found the maximum velocity of an unloaded single fibre from frog sartorius to be about 160 per cent of L_o/sec at 0° C. There are also great differences in velocity between different types of smooth muscle. The maximum velocity of mesenteric arteries, expressed as the maximum initial rate of an unloaded muscle at 38° C, was found to be about 0.25 per cent of L_o/sec (Lundholm and Mohme-Lundholm 1962). For uterine muscle load with a 2 g load (7 per cent of P_o) at 37.5° C, Csapo (1962) found maximum velocity to be 4.5 mm/sec, i.e. 14 per cent of L_o/sec; by extrapolation on his force velocity curve he calculated that the velocity of an unloaded muscle was 5.5 mm/sec, i.e. 17 per cent of L_o/sec. The maximum velocity of taenia coli under corresponding tensions were 10 per cent of L_o/sec and 30 per cent of L_o/sec (Fig. 1 A). Thus the form of the force velocity curve for taenia coli is different from that for uterine muscle.

In the experiments described in this paper the velocity of contraction was limited by diffusion processes (chemical stimulation) and by the rate of conduction of an action potential along the whole piece of muscle (electrical stimulation by single pulses). Higher velocities have been obtained when the muscles were subjected to high frequent electrical stimulation simultaneously over the entire muscle length. The maximum force was however the same.

We wish to thank associate professor Lennart Lundholm for stimulating discussions and various help, eng. Andras Bevia for construction of the transducers for isotonic measurements and Miss Lisbeth Olsson for valuable technical assistance.

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The Effect of Removing and Readmitting Glucose on the Electrical and Mechanical Activity and Glucose and Glycogen Content of Intestinal Smooth Muscle from the Taenia Coli of the Guinea Pig

By

J Axelsson S G R Hogberg and A R Timms[1]

Received 28 August 1964

Abstract

Axelsson J S G R Hogberg and A R Timms *The eff ct of emoving and readmitt ng glucose on the lect ical and mechanical activity and glucose and glycogen content of intestinal smooth mus le from the taenia coli of the guinea p g* Acta physiol scand 1965 64 28—42 The effects of cl anging the glucose concentration in the bathing solution on frequency of spike discharge membrane potential and on the mechanical responses is described quantitatively and related to tissue content of glucose and glycogen Removing glucose increases and readmitting inhibits spike discharge The membrane potential depends on carbohydrate metabolism for its maintenance Its dependence is however quantitatively or qualitatively different from that of the contractile mechanism Spike discharge of high frequency persisted for hours after the mechanical re sponse was abolished In glucose free solution the mechanical response fell in parallel with tissue content of glycogen In normal solution there was no correlation between tension and glycogen content

[1] Part f the biochemical work repr rted was done while one of the authors (A R T) was working in th Dept of Path bi logy Johns Hopkins Sch ol of Hygiene and Pul lic Health Baltimore Maryl nd

The experiments n which th glucose and glycogen levels were measured were perf rmed under isot nic cond t on So ne m asu rement kindly perf rmed for us under is metric c n dit ons by Dr F B1 d ng dem nstrate cl arly that glycog n d plet on is incompl t even after 2 hrs und r gluc se free nd t ons

	Glycogen µg mg w t weight Exp I	Exp II
Controls (2 hrs glucose)	0 84	1 14
45 min glucose fr e	0 54	0 98
2 hrs glucose fr e	0 9	0 69

(All values are mean f 3 1 t r inat n

The authors are ext m l g at f l t Dr Bued ng f r ca rying out these experiments

In 1912 Rona and Neukirch found that in glucose free solution the mechanical activity of intestinal smooth muscle diminished and disappeared These findings were confirmed and extended by Feldberg and Solandt (1942) who worked on the isolated small intestine of the rabbit

Working on taenia coli from guinea pigs Axelsson Bueding and Bulbring (1959) found that the abolition of mechanical activity in glucose free solution is not due to cessation of spontaneous discharge On the contrary the frequency of spontaneous discharge was increased on glucose removal and inhibited by returning glucose to the bathing solution In spite of continued electrical activity however the tension response was gradually abolished in the absence of glucose

The effect of glucose withdrawal and readmission has been examined in more detail Three aspects of this problem have received particular attention namely (A) the effects of removing and readmitting glucose on the electrical activity and the mechanical changes following the spikes (B) the effect of glucose on the mechanical response to supramaximal stimulation and (C) the effects of variations in external glucose on the glucose and glycogen content of this muscle

Methods

The tissue used was the taenia coli of the quinea pig For description of dissection pretreatment and determination of inactive in vitro length (Lo) see Axelsson and Åberg (1965) As we found that the glycogen level of this muscle changes significantly during the first 2 hrs in the isolated organ bath we adopted routinely a two hours period of incubation at 36 C under aerobic conditions before commenc ng any experimentation

S lutions The solution referred to as normal solution was a modified Krebs solution The compos tion was as follows (mM) Na 137 47 Ca^{+} 2 49 K 5 93 Mg 1 19 Cl 134 11 HCO_3^- 15 48 H PO_4^- 1 19 Glucose 11 5 It was aerated with a mixture of O (97) and CO (3) Its pH was 7 25

In the glucose f ee solut on glucose was replaced by equivalent amount of sucrose

The solution referred to as high potassium solution contained 12 8 m K This was obtained by replacing all the NaCl of the normal solution with KCl

Record n of electrical and mechanical activity For simultaneous recording of electrical and isometric mechanical activity we used the sucrose gap method (Stampfli 1954) For isometric recording of muscle tension we used two different types of transducers RCA 5734 and Grass type FT03 Isotonic measurements were obta ned with either a differential transformer or a mechano photoelectric transducer

St mulation Electrical stimulation was performed in similar way as described by Burnstock (1958) Chemical stimulat on was performed by rais ng the external concentration of potass um to 128 mM For further description of recording techn que and stimulation see Axelsson and Åberg (1964)

Determ nation of gl cos and glyc gen

Seven or eight p eces of muscle f om the same animal were subjected as far as possible to the same experimental conditions as a further piece of muscle from which electrical and mechanical activity and tens on were simultaneously recorded Samples were removed from the bath at app opriate times and their content of glucose and glycogen determined

Glucose The glucose content of the tissue was determined enzymatically (Slein Cori and Cori 1950) We ghed samples of taen a col muscle were removed from the bath and qu ckly frozen by placing them on a petri dish conta n ng dry ice The frozen pieces were then rap dly drawn through ice-cold glucose free Krebs solution so that glucose adherent to the outside of the tissue was washed off while the rema nder of the tissue remained frozen. The ex ess fluid was then removed by repeatedly drawing the tissue over a glass plate Drying the tissue with filter paper was avo ded because it was found that this resulted in a cons derable loss of glucose The weighed p eces were quickly immersed in chilled all glass homogenizers conta ning 0 4 ml of ice cold 4 perchloric acid and homogenized The homogenates were transferred to chilled centrifuge tubes and centrifuged at 4 C for 15 min at 1 000 × *g* Measured olumes of

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Part of the biochemical work reported was done while one of the authors (A R T) was working in the Dept of Pathobiology Johns Hopkins School of Hygiene and Public Health Baltimore Maryland.

The experiments in which the glucose and glycogen levels were measured were performed under isotonic conditions Some measurements kindly performed for us under isometric conditions by Dr E Bueding demonstrate clearly that glycogen depletion is incomplete even after 2 hrs under glucose free condition

	Glycogen μg/mg wet weight Exp I	Exp II
Controls (2 hrs + glucose	0 84	1 14
45 min glucose free	0 54	0 98
2 hrs glucose free	0 29	0 19

(All values are means of 3 determinations)

The authors are extremely grateful to Dr Bueding for carrying out these experiments

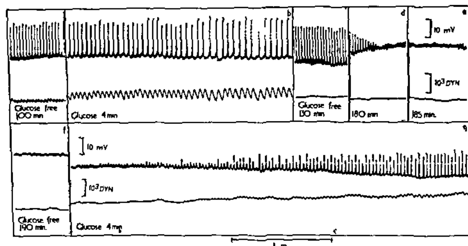


Fig 2 Reduction in membrane potential and abolition of spike discharge after prolonged exposure to glucose free solution and restoration of both by readmitting glucose Records as in Fig 1 For description see text

spontaneous spike discharge and on the tension response to each spike After 30 min exposure to glucose free solution the tension response had decreased by 66 per cent while the frequency of spike discharge had increased by 82 per cent After 120 min exposure the tension had decreased to 10 per cent of the tension in normal solution The frequency of spike discharge slowed again but was still 36 per cent greater than that in normal solution During 2 hrs exposure to glucose free solution the membrane potential decreased by about 10 mV

Glucose (11 5 mM) was readmitted after 122 min exposure to glucose free solution (*Fig 1B*) *In 24 sec spontaneous activity stopped and the membrane potential increased* After about 10 min in normal solution the first spike appeared followed by the development of tension which was 50 per cent of the normal At that point the membrane potential began to decrease slowly and within 12 min after readmitting glucose spontaneous spikes of the same frequency as in normal solution were recorded By this time the tension had recovered to 83 per cent of that recorded in normal solution at the beginning of the experiment

Qualitatively similar results were obtained with 40 muscles but the time taken to reduce the tension response by 50 per cent varied from 10 to 70 min

The maintenance of tension in glucose free solution was inversely related to the frequency of spike discharge

The initial effect of removing glucose from the bathing solution was always an increased frequency of spontaneous spike discharge After prolonged exposure a slowed time course of the compound spikes was observed

The ultimate effect of removing glucose was a fall in membrane potential and cessation of spontaneous spike discharge This occurred after 4—8 hrs exposure to glucose free solution Fig 2 illustrates the effect of long term exposure to glucose free solution In (a) the muscle had been in glucose free solution for 100 min The frequency of spike discharge was increased by 100 per cent and tension decreased by 90 per cent

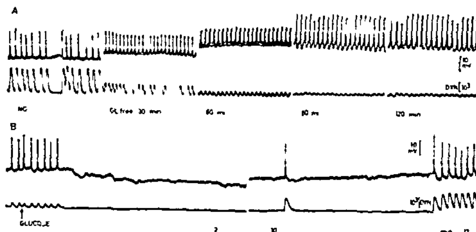


Fig 1 The effect of removing and readmitting glucose on spontaneous electrical and isometric mechanical activity A The effect of removing glucose B The effect of readmitting glucose Upper record electrical activity Lower record tension For description see text

the supernates were neutralized with 2 N KOH to pH 7 0 after the addition of 0 05 ml 0 5 M potassium glycyl glycine buffer (pH 7 4) Potassium perchlorate was removed by centrifugation at 100 × g for 15 min at 4 C Aliquots of the neutralized supernates were taken and glucose was determined in a reaction mixture containing 0 01 MgCl 0 05 M potassium glycyl glycine buffer (pH 7 4) 0 0003 M TPN and 0 003 M ATP in a final volume of 0 8 ml (Bueding *et al* 1960) 0 02 ml glucose-6-phosphate d hydrogenase (Boehringer) diluted 1 50 in 0 05 M potassium glycyl glycine buffer pH 7 4 and 0 02 ml of hexokinase (Boehringer) diluted 1 20 in the same diluent were then added to the cells and the increase in optical density at 360 mμ resulting from the formation of TPNH was measured spectrophotometrically The reaction usually went to completion in less than seven minutes

Glycogen Glycogen was determined in weighed samples of taenia coli by digestion in 0 2 ml of 45 KOH at 100° C for 0 min followed by precipitation at the same temperature with 3 volumes of 95 ethanol After allowing precipitation to occur (5 min at 4 C) the samples were centrifuged at room temperature at 3 000 rpm for 15 min and the clear supernatant fluid was discarded Glycogen was determined in the residue by the method of Bueding and Hawkins (1964) by degradation to glucose 1 phosphate and glucose using 1 6 amyloglucosidase and phosphorylase in the presence of inorganic phosphate and AMP Glucose 1 phosphate and glucose were then determined enzymatically

Results

A *The effect of removing and readmitting glucose on electrical and isometric mechanical activity*

1 *Spontaneous activity* Fig 1 A shows the effect of glucose removal on the frequency of

concentration modifies the spontaneous activity of taenia coli. Changes in frequency of spike discharge spike configuration and membrane potential, complicate the comparison of the mechanical responses to spontaneously arising spikes in glucose free with those in normal solution We tried therefore to eliminate some of the variables by applying electrical stimulation at fixed intervals in both normal and glucose-free solution. Electrical stimulation made it possible to compare the mechanical responses to stimulations of identical frequency strength and duration in normal solution with those obtained at different intervals after removal of glucose.

The device for electrical stimulation was similar to that described by Burnstock (1958)

The highest rate at which each muscle would respond to electrical stimulation ("driving rate) was determined in normal solution. The muscles were stimulated at this rate and at various lower rates. The rate at which the mechanical response was greatest was determined and is called the "optimum rate" The duration of each series of stimuli was 30 sec the intervals between stimulation lasted 10 minutes Duration and strength of stimuli was unchanged throughout the experiment. The optimum strength duration and frequency varied from one muscle to another Fig 3 (a) and (b) shows the mechanical response in normal solution both to spontaneously arising spikes and to electrical stimulation of two different frequencies (24 and 30 stimuli/min) 30 stimuli/min was the optimum driving rate in normal solution Parts (c) (d) and (e) show the decrease in the mechanical response to both spontaneous and electrically evoked action potentials in glucose free solution The frequency of stimulation shown in (c) is 30 stimuli/min. The driving rate was however greatly increased and (d) shows the response to 48 stimuli min After 60 min no tension response could be obtained either to spontaneous or evoked spikes (e)

In normal solution the maximum tension maintained by spontaneously arising spikes was similar to that in response to electrical stimulation of "optimum rate On a few occasions slightly higher tension could be obtained by electrical stimulation than was evoked by the spontaneously arising spikes after prolonged exposure to glucose-free solution However the time taken for the disappearance of the mechanical response to electrical stimulation was similar to that described for spontaneous activity

In (f) is shown the effect of readmitting glucose on both spontaneous activity and excitability Within 4 min spontaneous activity stopped and the muscle did not respond to electrical stimulation of the strength and duration which had previously been effective The membrane potential increased and spontaneous activity did not reappear until the muscle had been 12 min in normal solution (g) The frequency of spike discharge and the tension increased thereafter slowly (h) and (i)

B *The effect of removing and readmitting glucose on the mechanical response to supramaximal stimuli*

The mechanical response was recorded both isotonically and isometrically Isotonic recording was done with preloaded muscles under two constant loads 1 000 and 5 000 dynes The stimulation used in these experiments was high $[K^+]_o$ see methods The duration of each stimulus was 5 min The muscles were stimulated with 15 min intervals throughout each experiment.

In normal solution the muscles were spontaneously active When measuring the maximum mechanical response to changing from normal solution to a solution containing 128 mM K we either waited until the muscle was inactive or stimulated during

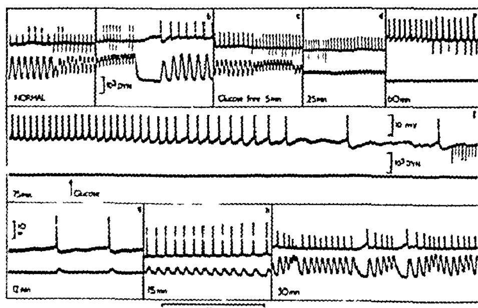


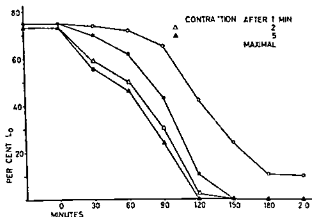
Fig 3 Effect of removal and readmission of glucose on spontaneous and evoked activity Records as in Fig 1 For description see text

Then glucose was admitted for 10 min In (b) the usual effects of glucose are seen decrease in spontaneous frequency of spike discharge and increased membrane potential and tension This time glucose did not completely abolish spike discharge as it did in Fig 1 after 120 min exposure to glucose free solution The glucose was again removed and panels c—f show the changes which took place First there was an increase in frequency thereafter there was a gradual fall in membrane potential decreasing amplitude and time course of spikes and finally small oscillations After a total of 4 hrs and 40 min in glucose free solution glucose (11 5 mM) was added once more The effects are shown in g As usual the glucose caused an increase in membrane potential At the time when glucose was readmitted the membrane was depolarized below the level of spike generation therefore an increase in membrane potential led in this case to a restoration of spike discharge Thus the effect of glucose in one and the same muscle may either be an inhibition or an initiation of spontaneous discharge depending on the membrane potential at the time of glucose administration

In all cases when glucose was readmitted at a stage when spike discharge was still present it decreased or abolished spontaneous spike discharge and caused an increase in membrane potential of varying degree In appearance the effect of glucose at this stage was indistinguishable from the usual effect of adrenaline on this muscle (Bulbring 1954

In most experiments in which glucose was readmitted when spikes still persisted one could observe a restoration of mechanical activity simultaneously with the inhibition of spike discharge The tension response to each spike began to increase and the frequency of spike discharge to decrease 30—50 sec after glucose was readmitted

2 *Electrical stimulation* The experiments described so far show how the external glucose



F g 5 The maintenance of isoton c shortening during st mulation in glucose free solution The muscle was under 5 000 dynes constant load Ordinate the amount of shortening at different intervals after onset of stimulation expressed as per cent reductions of the p eloaded L_O (at 5 000 dynes) Abscissa time in glucose-free solution

Expressed as percentage of the preloaded L_O the shortening of the muscles under 1 000 dynes load was decreased by only 15—20 per cent after 3 hrs exposure to glu cose free solution The maximum shortening of the muscles mounted under 5 000 dynes load was however decreased by 84—88 per cent at the same time

b *The effect on physical work*

In Fig 4A the physical work performed by four muscles is plotted against time in glucose free solution a and b were under 1 000 dynes load c and d under 5 000 dynes load The shortening of all four muscles in normal solution ranged from 65 to 75 per cent of L_O The physical work performed by a and b was however small compared with c and d which were under 5 times greater tension The physical work of a and b was only reduced by 15—20 per cent after 3 hrs exposure to glucose free solution The work performed by c and d was reduced more than 80 per cent

c *The effect on the du ation of contraction*

Although the physical work of muscles under 1 000 dynes load was only slightly de creased the ability of the muscles to maintain the contraction was greatly reduced

In Fig 4 (B and C) the whole course of contraction of two different muscles is shown The graphs show 15 and 1? records respectively at different stages during exposure to glucose free solution B was under 1 000 dynes C under 5 000 dynes load

This phenomenon receives more quantitative treatment in Fig 5 The amount of shortening (expressed as percentage reduction of the preloaded L_O) at various intervals after the onset of stimulation is plotted against time in glucose free solution The muscle was under 5 000 dynes load In normal solution the contraction remained almost unchanged during the 5 min of stimulation but in glucose free solution the contraction was not maintained

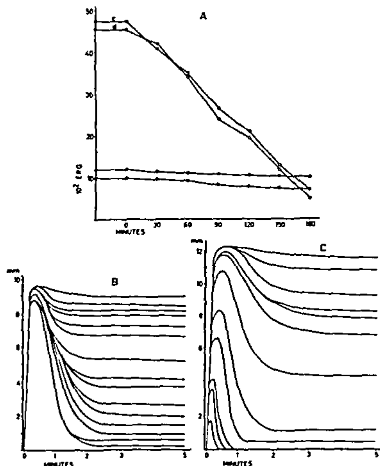


Fig 4 Effect of exposure to glucose free solution on physical work in response to stimulation with 128 mM [K] (A) and on contractures produced by the same potassium concentration (B and C) Abscissae the time in glucose free solution A Ordinate the physical work performed by 4 muscles a and b were under 1 000 dynes c and d under 5 000 dynes tension B and C Ordinates isotonic shortening B under 1 000 dynes C under 5 000 dynes tension The top curve in both B and C shows the concentrations in normal solution The successive curves were recorded at 15 min intervals in glucose free solution and show especially the decrease in the maintenance of contractures

a spontaneous contraction The results obtained by these two methods did not differ significantly

The initial effect of glucose removal was increased frequency of spontaneous activity resulting in shortlasting increase in muscle tone Usually after 30 min exposure to glucose free solution no spontaneous mechanical activity was recorded

1 *Isotonic recording*

a *The effect on the maximum shortening*

L_0 (the resting length of preloaded muscle was constant for the muscles which were mounted under 1 000 dynes load during 3 hrs exposure to glucose free solution L_0 of the muscles mounted under 5 000 dynes load increased by 4 5 per cent per hr

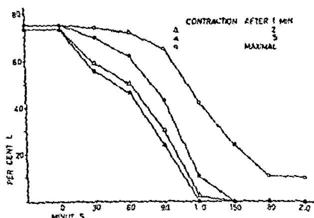


Fig 5 The maintenance of isotonic shortening during stimulation in glucose free solution The muscle was under 5 000 dynes constant load Ordinate the amount of shortening at different intervals after onset of stimulation expressed as per cent reductions of the preloaded L_O (at 5 000 dynes) Abscissa time in glucose-free solution

Expressed as percentage of the preloaded L_O the shortening of the muscles under 1 000 dynes load was decreased by only 15—20 per cent after 3 hrs exposure to glucose free solution The maximum shortening of the muscles mounted under 5 000 dynes load was however decreased by 84—88 per cent at the same time

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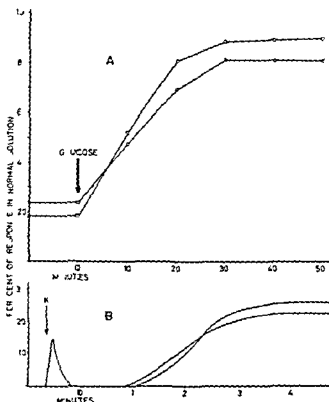


Fig 6 Recovery of mechanical response to h gh (K)ₑ Ordinates the sh ortenings obtained in each stimulation expressed as per cent of the maximum shortening in normal solution Abscissae t me in normal solution A and B isoton c shortenings under 5 000 dynes constant load B Th muscles had been n glucose free solution for 3 hrs At the beginning of the F gure they were stimulated as u ual with h gh (K) At 0 glucose (11 5 mM) was added to the h gh K solution

d The effect on the velocity of shortening

Under 1 000 dynes load the velocity of shortening in one muscle decreased from being 1 27 mm sec in normal solution to 0 83 and 0 37 mm sec after 1 respectively 2 hrs in glucose free solution

e The recovery of the mechanical response in normal solution

Fig 6A shows the time course of the recovery of the maximum shortening in response to supramaximal stimulation The maximum response of the two muscles examined was reduced to 19 and 24 per cent respectively of that in normal solution during 3 hrs previous exposure to glucose free solution These muscles were stimulated 10 min after readmitting glucose and then with 10 min intervals In 30 min their maximum response was restored to 80 – 90 per cent of normal

To obtain m re detailed information about the time course of the utilization of glucose by the c ntractile mechanism the following experiment was done

The muscles were exposed to 1 8 mM K after 3 hrs exposure to glucose free solution When the initial shortlast ts response was over glucose was added while the muscle

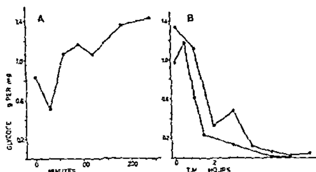


Fig 9 A The rate of glycogen synthesis in normal solution — see text
B The fall in the glycogen content of the muscles in glucose free solution

content rose to 50 per cent of the initial pre-depletion level and in three minutes the level was approximately 75 per cent of the initial Thereafter the rate of repletion decreased and in fact the results obtained suggest that the initial pre-depletion level is not completely reattained at least within the 15 min duration of these experiments

In the control muscles the electrical and mechanical activity in normal and glucose free solutions was identical with that described in section A

2 *Changes in the glycogen content of taenia coli*

Fig 9A illustrates an experiment in which the glycogen was determined at various intervals after the muscle had been mounted in normal bathing solution under 1 000 dynes resting tension

Although this represents only one experiment, the results suggest that the glycogen level of this tissue changes significantly after it is set up in the organ bath After 2 hrs glycogen was increased by approximately 35 per cent compared to a sample taken immediately before setting up Thereafter the level of glycogen appeared to increase slowly

3 *The effect of glucose withdrawal on the glycogen content*

The present studies indicate that under glucose free conditions the fall in tissue glucose levels was not parallel to the fall in tension The possibility existed, however that the degradation of glycogen was responsible for the maintenance of tension under glucose free conditions Accordingly the rate of depletion of glycogen was determined under glucose free aerobic conditions

Fig 9B shows the results of 2 expts The rate at which glycogen disappeared from the tissue is relatively slow After one hour the fall in glycogen ranged from 15 to 50 per cent Even after 4 hrs there was still a significant amount of glycogen present in the tissue In these experiments practically no glycogen could be detected after 6 hrs exposure to glucose free medium However Bueding and Hawkins (1964) using a more refined version of the original method (which was used in the present experiments) found that even after six hours under glucose free aerobic conditions significant amounts of glycogen could be detected It should be noted however that the initial more rapid fall in glycogen appears to have essentially the same time course as the loss of the mechanical response

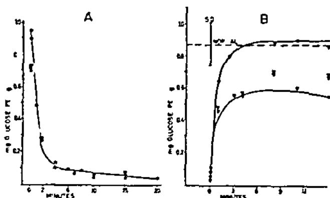


Fig 8 A Rate of depletion of tissue glucose from taenia coli under glucose free conditions. Values taken from 4 expts from different animals B Repletion of tissue glucose in taenia coli depleted of glucose in glucose free medium 10—30 min at 3C C (4 expts) After transfer to normal solution samples were removed at various time intervals Shaded area indicates scatter of values Normal mean glucose level and S D shown

normal was obtained in all muscles within 1 hr The time course of recovery however differed considerably Few muscles recovered to 80—90 per cent of normal response in 30 min

In a few experiments after prolonged exposure to glucose free solution adrenaline was added to the bathing solution before stimulation It always caused a shortlasting increase in tension or physical work in response to stimulation

C *The effect of removing and readmitting external glucose on the glucose and glycogen content of the muscles*

In all experiments 8 pieces of taenia coli were removed and subjected to identical experimental conditions One piece was used for recording spontaneous electrical and mechanical activity The remaining pieces were removed at intervals and their content of glucose and glycogen was determined

1 *Changes in glucose content of taenia coli*

The normal total tissue glucose level ranged from 0 7—1 1 mg/g wet weight The mean value was 0 87 mg/g with a standard error of ± 0 05 When the muscles were transferred to a glucose free solution a rapid fall in total tissue glucose occurred This is illustrated in Fig 8A Over 75 per cent of the glucose disappears in the first 3 minutes and thereafter the decline is much slower (only a further 10 per cent or so in the next 30 min) Control pieces kept in normal solution throughout the period of these experiments showed no significant change in their glucose content compared with the initial samples

The muscles from which the electrical and mechanical activity were recorded showed no decrease in mechanical activity during the first ten minutes in glucose free solution The characteristic initial increase in the frequency of spontaneous spike discharge occurred however during the period of the rapid fall in tissue glucose It is clear that the glucose levels would be extremely low at the time that dissociation of electrical and mechanical activity occurred

When glucose is readmitted after 10 or 30 min exposure to glucose free conditions the total tissue glucose levels rise very quickly (Fig 8B In the first minute the glucose

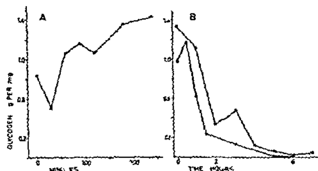


Fig 9 A The rate of glycogen synthesis in normal solution — see text.
B The fall in the glycogen content of the muscles in glucose free solution

content rose to 50 per cent of the initial pre-depletion level and in three minutes the level was approximately 75 per cent of the initial Thereafter the rate of repletion decreased and in fact the results obtained suggest that the initial pre-depletion level is not completely reattained at least within the 15 min duration of these experiments

In the control muscles, the electrical and mechanical activity in normal and glucose free solutions was identical with that described in section A.

2 *Changes in the glycogen content of taenia coli*

Fig 9A illustrates an experiment in which the glycogen was determined at various intervals after the muscle had been mounted in normal bathing solution under 1 000 dynes resting tension

Although this represents only one experiment the results suggest that the glycogen level of this tissue changes significantly after it is set up in the organ bath After 2 hrs glycogen was increased by approximately 35 per cent compared to a sample taken immediately before setting up Thereafter the level of glycogen appeared to increase slowly

3 *The effect of glucose withdrawal on the glycogen content*

The present studies indicate that under glucose free conditions the fall in tissue glucose levels was not parallel to the fall in tension The possibility existed however that the degradation of glycogen was responsible for the maintenance of tension under glucose free conditions Accordingly the rate of depletion of glycogen was determined under glucose free aerobic conditions

Fig 9B shows the results of 2 expts The rate at which glycogen disappeared from the tissue is relatively slow After one hour the fall in glycogen ranged from 15 to 50 per cent Even after 4 hrs there was still a significant amount of glycogen present in the tissue In these experiments practically no glycogen could be detected after 6 hrs exposure to glucose free medium However Bueding and Hawkins (1964) using a more refined version of the original method (which was used in the present experiments) found that even after six hours under glucose free aerobic conditions significant amounts of glycogen could be detected It should be noted however that the initial more rapid fall in glycogen appears to have essentially the same time course as the loss of the mechanical response

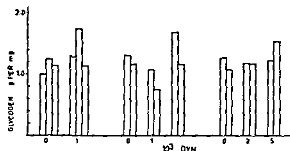


Fig 10 The glycogen content of muscles mounted under various tensions

Although glycogen is apparently utilized by taenia coli for maintenance of tension under glucose free conditions it does not necessarily follow that glycogenolysis is essential to this tissue under optimal conditions Fig 10 illustrates 3 expts in which a comparison of the glycogen content was made with samples of tissue from the same animal under different tensions It can be seen that no correlation exists between higher tension and lower glycogen In fact in all 3 expts the glycogen content under tension appeared not to differ significantly from that of muscles suspended in the same bath but with no tension

Discussion

Having established standardized conditions which permit quantitative treatment of the various mechanical parameters (Axelsson and Åberg 1965) the next step was to try to relate these to some metabolic processes which may ultimately provide the energy of contraction In the present work we have confined ourselves to the description of the relationship between various mechanical and electrical properties and the tissue content of glucose and glycogen

Physical work and tension

In normal solution there appeared to be no correlation between the tension under which the muscles were mounted and their content of glycogen (Fig 10) This suggests that glycogenolysis is not important to the maintenance of tension when glucose is present However final proof of this point must await evidence of the rate of turnover of glycogen for it may be that under higher tension the incorporation of glucose into glycogen is accelerated as well as the breakdown of glycogen In the absence of glucose however a parallelism between the fall in glycogen and the change of certain mechanical parameters becomes obvious Supporting evidence for the view that glucose may be utilized directly without conversion to glycogen comes from the fact that glucose is immediately utilized in the development of contractures by depolarized muscles which have lost their mechanical response in glucose free solution (Fig 6B) as well as in the development of tension in response to spikes (p 36) This suggests that under glucose free conditions glycogenolysis may be important for the maintenance of tension and this is borne out by the results of Bueding and Hawkins 1964) who demonstrated that

under glucose free conditions glycogen is depleted more rapidly and more completely when the muscles are subjected to a high degree of stretch than when they are stretched to a lesser extent In normal solution glucose may be the principal source of energy for the contractile processes

Maximum physical work is performed by this muscle at about 30 per cent P_O (the maximum force developed by the muscle at L_O isometric) (Axelsson and Åberg 1965) The initial shortening i e the physical work of muscles mounted under 1 000 dynes tension was only slightly affected by three hours exposure to glucose free solution (Fig 4A) It should be noted that during maximum shortening the work performed was only about 10^3 erg These muscles can continue to perform work in the absence of glucose but they are unable to maintain the contracture This ability (Fig 4B) fell in parallel with the glycogen content in glucose free solution (Fig 9B) Two muscles mounted under five times higher tension (5 000 dynes) (Fig 4A) performed 4 to 5 × 10^3 erg in normal solution Under these conditions the work was progressively decreased in glucose free solution The maintenance of contractures however was again affected much sooner than the physical work (Fig 5)

The isometric tension response appeared to decline more rapidly in glucose free solution than did the physical work (Fig 4 5 and 7) In this case the level of maintained tension fell simultaneously with the maximum tension response

Influence of metabolism on the electrical activity

Changes in the glucose concentration in the bathing solution were immediately reflected in the glucose content of the muscles (Fig 8) Simultaneously the frequency of spontaneous spike discharge was affected (p 11) When the glucose content fell the frequency increased When it rose spike discharge was inhibited In normal solution glucose appears to function as a brake on the frequency of spontaneous discharge

During prolonged exposure to glucose free solution the membrane potential gradually fell (Fig 2) At that time the glycogen content of the muscle was very low (Fig 9B) Thus the membrane potential might depend for its maintenance on carbohydrate metabolism The dependence of the membrane potential is however either quantitatively or qualitatively entirely different from that of the contractile mechanism It was possible for spike discharge to persist at high frequency for hours after the mechanical response was abolished (p 4) When glucose was readmitted after prolonged exposure to glucose free solution at a stage when spike discharge had ceased it caused a great increase in membrane potential (Fig 2) After shorter exposure glucose also caused an increase in membrane potential It might however be argued that the small increase in membrane potential seen for example in Fig 1 was secondary to the inhibition of spike discharge That argument does not apply to Fig 2 as no spike discharge persisted at the time of the readmission of glucose On the contrary glucose restored spike discharge at appropriate level for firing only to abolish it later as the membrane repolarized further

No specific membrane effect of glucose is known Besides various substitutes for glucose such as acetate and sodium acetoacetate have been found to have the same inhibiting effect on spike discharge as glucose (Axelsson Bulbring and Krebs unpublished) At the same time as glucose decreased the frequency of discharge it caused an increase in the force of the mechanical responses It is likely that the chief effect of glucose is to provide an increased supply of energy

All the effects described above are consistent with the view that variations in the energy supply derived from carbohydrate metabolism cause variations in the membrane potential and thereby in the frequency of spike discharge. The underlying mechanism may be (1) variations in the rate of active ion transport (2) a more direct dependence of the permeability constants of the membrane on energy supply or (3) a combination of both.

The striking similarity was pointed out (p 5) between the usual effect of adrenaline on the electrical activity of this muscle (Bülbring 1954 1956 Burnstock 1958) and the effect of glucose. This supports the view that the inhibiting effect of adrenaline on spontaneous spike discharge may be related to its metabolic stimulating action. Adrenaline has been found to cause a great increase in energyrich phosphate compounds (Bueding *et al* 1962) and even after prolonged exposure to glucose free solution adrenaline was found to increase the mechanical response to high [K]$_o$ in much the same way as does readmission of glucose for a short time (p 11)

Fig 2 illustrates that increased energy supply may have what appears to be opposite effects. In one and the same muscle readmission of glucose may either inhibit spike discharge or initiate it. Both effects are probably due to increased membrane potential what is observed depends on the membrane potential at the time of readmission. The action of a drug may be reversed in one and the same piece of muscle depending on its nutrition and the metabolic supply of energy at the moment of application. Thus it may not always be necessary to postulate different receptor sites for apparently opposite actions of one and the same drug. To do so simply restates the problem and does not necessarily help to explain it.

We are grateful to ing Andras Beviz, Miss Lisbeth Olsson and Mr A. Salama for skilful technical help and to the Swedish Natural-Science Research Council for financial support

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Auditory Activity in Uncrossed Centrifugal Cochlear Fibres in Cat

A Study of a Feedback System II

By

Jorgen Fex

Received 4 September 1964

Abstract

Fex J *Auditory activity in uncrossed centrifugal cochlea fibres in cat A study of a feedback system II* Acta physiol scand 1965 *64* 43—57 — In decerebrate cats all of the cochlear efferents except the uncrossed olivo-cochlear fibres were cut the vestibulo-cochlear anastomosis was exposed through the vestibule and single unit activity was recorded in and close to the anastomosis with micropipette electrodes Although the auditory function was destroyed in the dissected cochlea resting activity was found in primary auditory afferents suggesting that synaptic mechanisms could still be intact in such a preparation — Uncrossed olivo-cochlear fibres were activated by electrical stimulation of the ipsilateral cochlear nerve and by acoustic stimulation of the contralateral ear When activated by sound the uncrossed efferents in the basal fascicle of the anastomosis generally responded to higher tone frequencies than did fibres in the apical fascicle which suggests that afferents and uncrossed efferents from homotopic cochlear points in opposite cochleae are connected The fibres responded characteristically with a low regular firing rate without an initial burst and with a long latency at threshold indicating that their input would be integrated over a considerable time Many of the efferents showed resting activity which with few exceptions could be inhibited by sound depending on the choice of tone frequency — The findings suggest that the uncrossed cochlear efferents and ipsilateral auditory afferents together form an auditory closed feedback loop which is directly influenced by the contralateral ear and support the hypothesis (Fex 1962) that the uncrossed and the *crossed olivo-cochlear bundles together form a complex auditory feedback mechanism*

Two separate centrifugal tracts to the cochlea are known the uncrossed and the crossed olivo-cochlear bundles The crossed olivo-cochlear fibres have been studied rather extensively The fibres arise in the superior olivary complex (Rasmussen 1946 1953 1960) and form a large number of endings in the contralateral organ of Corti on hair cells and on afferent nerve terminals as shown by Iurato (1962) in the rat by Kimura and Wersall (1962) in the guinea pig by Smith and Rasmussen (1963) in the chinchilla and by Spoendlin and Gacek (1963) in the cat These crossed olivo-cochlear fibres are

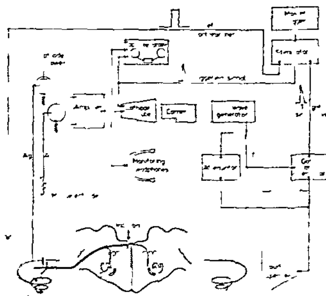


Fig. 1. Diagram of the experimental arrangement for stimulating and recording from the uncrossed olivo-cochlear fibres. COC, crossed olivo-cochlear fibres. RE, reticular efferents of Rossi and Cortesina (1962). SO, superior olivary complex. UCO, uncrossed olivo-cochlear fibres. VCA, vestibulo-cochlear anastomosis.

activated by sound and form part of an auditory feedback system as shown by Fex (1962) in a study of single cochlear fibres in the modiolus of the cat. The function of this feedback system is not well understood.

Much less is known about the uncrossed tract. It was first described in the cat by Rasmussen (1960) who traced the fibres from the S-shaped olivary segment into and through the vestibulo-cochlear anastomosis and estimated that the number of fibres were about 125. The fibres probably end in the organ of Corti but the exact termination is not yet known (cf. Spoendlin and Gacek 1963). Only one study has dealt with the functional significance of the uncrossed bundle. Thus it has recently been shown with a macroelectrode technique that electrical stimulation of the uncrossed olivo-cochlear fibres depresses the action potential component of the round window response to a click. Desmedt and LaGrutta (1963) found this effect to be considerably smaller than that of the crossed olivo-cochlear efferents (Galambos 1956).

In the present work it will be shown that the uncrossed olivo-cochlear fibres are also activated by sound.

Technique and procedure

Preparation. The present experiments were carried out on 40 cats free from ear infection and weighing between 1.5 and 3.7 kg. The cats were decerebrated precollicularly by suction. The initial stage of the operation was performed under ether anesthesia lasting 10—15 min. Subsequently, in order to prevent elevation of the blood pressure, a short lasting barbiturate (Thiogenal, E. Merck) in doses of 10—20 mg/kg was administered i. v. until 1 hr or more before the actual recordings.

The technique and the p ocedure used in this \ ork were to a large extent identical \ ith those described n an earlier paper (cf Fex 196? pp 8—16) The vest bulo-cochlear anastomosis \ as exposed on the left side Through a posterior craniotomy part of the cerebellum was removed to expose the fourth ventricle In order to cut all the efferents to the cochlea except the uncrossed olivo-cochlear fibres (cf Rasmussen 1946 Rossi and Cortesina 19(?) an incis on was made in the floor of the fourth ventricle (Fig 1) The incis on was made 0 3—0 5 mm to the left of and parallel with the m dline 1 mm deep and 4 mm long with the midpoint at the level of the facial genua The right ear was amputated

In a series of 16 animals stimulating electrodes were placed in the cochlear nerve peripheral to its basal turn This necess tated enlargement of the opening of the vestibular floor In these animals stimulating electrodes were also placed stereotaxically in d fferent regions in the left s de of the brain stem in order to stimulak the uncrossed olivo-cochlear fibres electr cally The elec trodes formed an angle of 45 with the horizontal plane pointing in a rostro-caudal direction The bony tentorium was thus avoided

Gallamine triethiod de (Flaxedil May & Baker) was used routinely in doses of 8—10 mg/kg to immobilize the cats in which electrical stimulation was used these cats were then artificially respirated Any tendency to develop edema of the brain was effectively countered by the ntravenous nject on of a 30 urea solution with 10 invert sugar in 3 of 4 doses of 0 30—0 40 g/kg at intervals of 1—2 hrs (see Javid and Settlage 1956 Javid 1958) When stimulating electrodes were not placed in the brain stem and Flaxedil and artificial respiration were not used brain edema was rarely seen and urea was not necessary In these cats the re cording cond tions were relatively stable. Some units were kept for more than 15 min No attempts were made to immobilize the recording site in any of the cats

The vestibulo-cochlear anastomosis was carefully dissected following each experiment as the final step in the attempt to decide to wh ch part cular fascicle each recorded unit did belong

There were no systemat cally recorded observations pertaining to the general condition of the animal The activity of the right ear was not mon tored in any d rect way

Electrical stimul tion The stimulator used had an output of 1 000Ω and delivered square wave pulses of 0 2—0 3 msec as single shocks or as bursts of repetitive stimuli The pulse form was distorted by the isolation transformer For stimulation of the left cochlear nerve peripheral to its basal port on two insulated copper wires with a diamete of 40μ were used the bared tips being 0 5 mm long and 0.5 mm apart f om each other and also from the recording s te in the vest bulo-cochlear anastomos s For st mulation in the S-shaped ol vary nucleus and in adja cent structures concentric electrodes \ ere used The inner electrode consisted of an enamalled plat num iridium wire with a diameter of 0 3 mm Its bared tip protruded 0 5 mm from the rim of the outer electrode consisting of an uninsulated steel tube with an outer d ameter of 0 8 mm

The stimulus strength used as expressed in voltage output of the stimulator varied between 0 8 V and 8V 2V being the strength generally used Stimulat ng current was not measured The stimulat on s tes were marked by electro-coagulation and subsequently identified in histological section

Acoustic st m lation The experimental devices used for acoustic stimulation in this series have been described previously (Fex 196? pp 11—12) To summarize (F g 1) the output from a tone generator was fed through attenuators to a loudspeaker either directly or through an electronic gate The gate was used to produce tone pips that were synchronous with the signal that triggered the sweep to the oscilloscope Continuous tones p ps or cl cks were produced and p esented in an open field through a dynamic loudspeaker placed 30 cm from the right tympan c membrane The dyn mic loudspeaker \ as calibrated under experimental condit ons The f equency response for the loudspeaker system w s flat within ± 6 db between 200—5 000 c/s and showed a few dips of the o der of 15 db between 5 000 and 7 000 c/s The magni tud and locat on of these dips d ffered w th d fferent pos tions of the loudspeaker with n experimental condit ons Above 7 000 c/s there was a downward slope of the order of 30 db/octave The loudspeaker s maximal output was 115 db SPL (SPL = Sound Pressu e Level rela tive 0 000? dyn/cm) Long tone pips we e used in the determinations of best f equenc es and of band w dths No correction has been made fo the loudspeaker system s deviat on from an ideally flat curve For latency measurements a hearing a d rece er was placed close to the right tympanic membrane mounted in one end of a 9 mm long tube the other end f ced the tympanic membrane at a distance of 2—3 mm

The figures for the nois level of the experimental room have been gi en prev ously (Fex 196? p 12) The record ng technique (F g 1) was ident cal with that described p eviously (Fex 196? pp 1?—13) exc pt that the capillary microelectrodes wer filled w th 3 M KCl

The change of noise in the monitoring loudspeaker and the presence of resting activity or activity evoked through stimulation with various sound stimuli were used as guides when probing with the electrode in the vestibulo-cochlear anastomosis. This method favours a high incidence of fibres with resting activity and a low incidence of fibres that respond to stimulation of the ipsilateral cochlear nerve only.

Results

As will be discussed below the activity recorded in the vestibulo-cochlear anastomosis in the present experiments could derive either from uncrossed olivo-cochlear fibres or from primary auditory afferents. Irregular resting activity considered to be afferent (see Discussion) is mentioned below. All remaining activity described here is considered to be efferent. A total of 189 uncrossed olivo-cochlear fibres were found in the 40 cats.

Attempts to identify the uncrossed olivo-cochlear fibres through electrical driving from the superior olivary complex and adjacent structures were not satisfactory because it proved very difficult to rule out the possibility that primary auditory neurons were antidromically driven through such stimulation. The findings of Lewy and Kobrak (1936) that primary auditory fibres project in a small number in the trapezoid body (see also Ramón Y Cajal 1952) offer an anatomical basis for this possibility, together with the findings of Rasmussen (1953) that a small number of primary auditory neurons may be found in the vestibulo-cochlear anastomosis. However, a central projection of primary auditory neurons beyond the cochlear nuclei has been denied more recently (Stotler 1949, 1961, Powell and Cowan 1962, also *cf.* Powell and Erulkar 1962).

Resting activity. 74 fibres out of 189 uncrossed olivo-cochlear fibres showed resting activity. This activity never occured in bursts and had a frequency of 3—50 imp/sec. As a rule the firing was regular except when the rate was very low. The activity of all fibres showing this kind of resting activity could be influenced by stimulation of the contralateral ear with sound or by electrical stimulation of the ipsilateral cochlear nerve.

Many fibres showing fast irregular resting activity were found deep below the surface of the vestibulo-cochlear anastomosis. It was not possible to decide whether these belonged to the anastomosis or not. The activity of these fibres was not changed by stimulation of either of the cochlear nerves and the fibres were held to be primary auditory fibres (see Discussion). Similar activity was recorded from primary afferents both close to and far away from the vestibulo-cochlear anastomosis.

Response to sound. Almost all uncrossed olivo-cochlear fibres responded to sound stimulation of the contralateral ear and showed excitation, inhibition or both. The mode in which a fibre showing both excitation and inhibition responded depended primarily on the frequency of the stimulating tone. Many of the 189 fibres were lost before it could be ascertained whether or not they could be both excited and inhibited by sound.

a. The excitatory effect of sound to the contralateral ear was tested in 184 out of the 189 uncrossed olivo-cochlear fibres and of these 177 fibres showed excitation. Of the remaining seven fibres, five showed inhibition only and two fibres did not respond to sound but only to electrical stimulation of the ipsilateral cochlear nerve.

Uncrossed olivo-cochlear fibres that were excited by sound showed with very few exceptions no initial bursts and had a regular and low firing rate (Fig. 2). These characteristics were shared with the crossed olivo-cochlear fibres (*cf.* Fex 1962). Most fibres had a graded response to sound and of the 20 fibres tested in this respect 18 had a dynamic range between 30 and 60 db. The firing rate of one fibre did not reach a

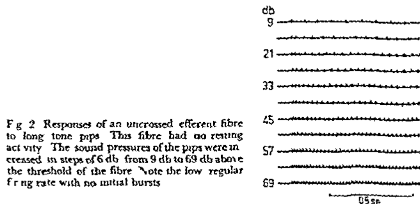


Fig 2 Responses of an uncrossed efferent fibre to long tone pips This fibre had no resting activity The sound pressures of the pips were increased in steps of 6 db from 9 db to 69 db above the threshold of the fibre Note the low regular firing rate with no initial bursts

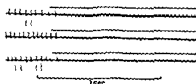


Fig 3 Inhibition by sound of the background activity in an uncrossed efferent fibre Three consecutive tone pips of 2 sec duration with intervals of 2 sec were given as indicated by the horizontal bars Note that the inhibition decreases with repetition

plateau within the 54 db over which it could be tested The firing rate of the unit of Fig 2 was still increasing with increasing sound pressure at 69 db above threshold

The firing rates illustrated in Fig 2 are representative However some fibres could not be stimulated to discharge more than 5—10 impulses/sec while the highest firing rate at the beginning of a train of impulses was 12 impulses during 100 msec The shortest spike interval produced by short strong pips of sound was 4 msec

Some fibres showed a pronounced decrease in firing rate with time in response to loud continuous tones The resting activity in eight of the fibres was silenced for some seconds after loud tone pips of long duration 6 out of these 8 fibres could be inhibited by sound the remaining 2 fibres were not tested in this respect In five fibres with no resting activity there was an after-discharge for several seconds following sound stimulation

b Inhibition of resting activity in response to sound (Fig 3) was tested in 44 fibres out of the 189 uncrossed olivo-cochlear fibres and was found in all but five 2 out of these 5 fibres could not be excited by sound a third fibre was only excited at very high sound pressures and a fourth showed an uncommon instability of the resting activity In these 4 fibres brain stem electrodes may have interfered with the input 34 fibres out of the 39 that could be inhibited were tested also for excitation 5 out of these 34 could not be excited but on the other hand all five were inhibited within almost the total range of tone frequencies employed In the 4 cats corresponding to these 5 units brain stem electrodes were not used

A reduction of inhibition was seen at high pressures of sound Rebound after inhibition was never observed

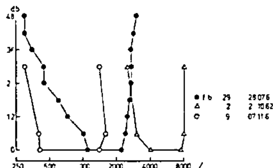


Fig 4 Sound pressure curves for three uncrossed efferents tested for ranges of frequencies which gave excitation at different sound pressures increasing in steps of 6 db or more relative to the thresholds of the neurons The different values of the absolute thresholds are not given Note the wide range of tone frequencies giving excitation at the respective excitatory thresholds

Thresholds The lowest sound pressure using steps of 3 db at which a fibre could be excited or inhibited will be called the fibre s excitatory or inhibitory threshold respectively The tonal sine frequency at which the threshold was found will be referred to as the fibre s best excitatory (see Fex 1962) or inhibitory frequency

*Every unit that could be tested in this respect had a well defined excitatory or in*hibitory threshold or both with the qualification that strong sounds often increased the threshold

The absolute values for the excitatory thresholds of 109 fibres out of which 37 fibres had shown resting activity were calculated The median for this population was about 50 db SL. Several fibres were excited at approximately 20 db SPL but on the other hand quite a few fibres did not respond below sound pressures of 80 db SPL

An analysis of the results did not indicate that fibres with resting activity had lower thresholds than those without such activity The findings instead expressed the marked tendency that the higher the incidence in a given experiment of fibres with resting activity the lower were the excitatory threshold of the fibres One of several obvious explanations of these findings is that small changes in the height of the precollicular decerebration had a decisive influence on the activity of the uncrossed olivo-cochlear efferent system

For 12 fibres that showed both excitation and inhibition the respective absolute thresholds could be determined The excitatory threshold was lower than the inhibitory threshold in all instances but one and the differences ranged from — 6 db to 54 db The lowest inhibitory threshold found was at 40 db SPL

Best frequencies and band width The uncrossed olivo-cochlear fibres generally responded over a wider range of tone frequencies at the excitatory threshold than did the crossed efferents most of which had best frequencies determinable within 10 % The sound pressure curves of Fig 4 give a graphical expression of the frequency range at different sound pressures for three different fibres When discussing such fibres or many of the other uncrossed efferents it would be more appropriate to refer to best excitatory band instead of best excitatory frequency However some uncrossed efferent fibres had a best excitatory frequency which could be determined within 10

Sometimes it could be decided in which fascicle of the vestibulo-cochlear anastomosis the electrode tip had been placed Thus it was possible to ascertain whether or not fibres running to different parts of the cochlea responded preferentially to different frequencies Table I offers data on all fibres which ran in the most basal or the most apical fascicle of the vestibulo-cochlear anastomosis as verified at section and for which

TABLE I

		Fibres from the most apical fascicle			Fibres from the most basal fascicle		
Cat	Fibre	Best frequency	Best frequency for inhibition	Frequency range	Best frequency	Best frequency for inhibition	Frequency range
18 07 62	1	1 500					
25 07 62	7				6 000	3 000	
23 10 62	2	5 000		1 000—8 000			
01 11 62	1	900—1 500	2 200				
12 11 62	1a				3 300		
	1				1 200—1 300		250—3 000
	2	800					
	3	700					
	8				4 500—5 200		400—10 000
	10	1 200—1 900					
13 11 62	1				8 000—9 000		
	3	800—950		250—1 500			
16 11 62	1				8 000—8 500		700—13 000
	2				6 300—6 900		
20 11 62	2				5 000—5 500		650—8 000
	3				5 800—6 200		650—8 200
	4				4 900—5 400		900—6 [illegible]00
2[illegible] 11 62	1	1 700					
	2	710—870		200—3 000			
23 11 62 I	1	800		350—1 500			
	2	1 000	1 [illegible]00–2 500				
	6	800—1 300	1 900–2 [illegible]00				
23 11 62 II	1				15 000—16 000		
	2				20 000		
	3				9 000—11 000		6 500—13 000
	4				9 000—9 500		
24 11 62	4	500	1 500				
26 11 62	1				9 000		
					6 600—7 000		
	3				15 000—20 000	3 000–3 500	
28 11 6[illegible]	1		3 000				
	2		3 000				
	3	[illegible]30—830		[illegible]00—1 800			
	5	700—800		[illegible]00—2 200			
		700—850					
30 11 62	1				6 000—[illegible] 000		

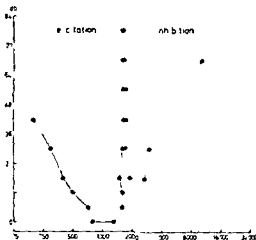


Fig 5 Sound pressure curve for an uncrossed efferent fibre tested for ranges of frequencies giving excitation and inhibition at different sound pressures The sound pressures increased in steps of 6 db at first then 12 db relative to the excitatory threshold Note change from excitation to inhibition within a narrow frequency range around 1550 cps at high sound pressures (Cat 23 11 62 I fibre 6)

best frequencies or best frequency bands had been determined In some instances best frequencies are given as approximations for best frequency bands

The best frequencies from the basal fascicle were higher than those from the apical fascicle with only two instances of overlapping (Table I unit 2 cat 23 10 62 and unit I cat 12 11 62)

Resting activity in fibres from the apical fascicle was inhibited by tones of frequencies higher than the best excitatory frequency in the four units with full information on this point On the other hand in two corresponding units from the basal fascicle resting activity was inhibited by tones of frequencies lower than the best excitatory frequencies Table I)

Sound pressure curves were determined for only very few fibres which showed inhibition The characteristics of such a fibre are displayed in Fig 5 Although the fibre's frequency discrimination was poor at the respective thresholds it was very good at high sound pressures in that excitation of the fibre was changed into inhibition with a change of tone of only 100—50 cps around 1 550 cps

Primary auditory neurons with best excitatory frequencies within the lower part of the audio frequency range have as a rule a symmetrical response area (Katsuki *et al* 1962 when excited by sound of different frequencies and at different pressure levels In other words the sound response curves of these neurons do not show a cut-off at the high frequency side A high frequency cut-off however characterizes primary auditory neurons with high best frequencies (*cf* Katsuki *et al* 1962) and secondary auditory neurons Galambos and Davis 1943 Tasaki and Davis 1955 Katsuki *et al* 1958 Rose *et al* 1959 Crossed olivo-cochlear fibres responding to high frequencies (Fex 1962 often lacked a high frequency cut-off In the present experiments many uncrossed olivo-cochlear fibres with no high frequency cut-off were found from all parts of the vestibulo-cochlear anastomosis within the low as well as within the high frequency range Some of these results are expressed in Table I in which frequency range denotes the range within which the respective fibres were excited at the maximal output of the loudspeaker system

Latency of response to sound Latency determinations were made for 17 fibres excited by tone pips The latency of many units was found to depend markedly on the sound

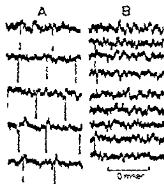


Fig 6 Inhibition by sound of background activity in a single uncrossed efferent fibre A the background activity B tone pips were presented starting synchronously with the triggering of the sweeps and inhibited the background activity (see text)

pressure used A typical unit gave the following findings When the sound pressure was high the latency was 8—9 msec when the pressure was just above threshold the latency was 40 msec and at an intermediate sound pressure the latency was 15 msec In some fibres considerably longer latencies could be found at sound pressures close to thresholds

Latencies between 5 and 20 msec were found in the 27 fibres when high sound pressures and short tone pips were used give to the shortest possible latencies 22 of the fibres had latencies of 5—10 msec

Although the latency for inhibition of resting activity has not been studied closely in this work one of the shortest latencies for inhibition found in these experiments is illustrated in Fig 6 The resting activity (Fig 6 A) was inhibited about 10 msec after the tone pips were presented at the beginning of the sweep (Fig 6B) In a series of 150 consecutive sweeps no spike occurred later than 9 msec after the start of the tone pip

Response to electrical shocks to the ipsilateral cochlear nerve It was shown above that uncrossed olivo-cochlear fibres respond to acoustic stimulation of the contralateral ear The ipsilateral ear could not be used for testing by physiological stimulation since the cochlea had been put out of function as part of the preparation for the experiment However the cochlear nerve could be stimulated electrically In an attempt to avoid antidromic stimulation of the uncrossed olivo-cochlear fibres, the portion of the cochlear nerve lying peripheral to the basal turn was chosen for stimulation *i e* the apical part. Electrical stimulation of the cochlear nerve was carried out satisfactorily in ten out of the sixteen animals Single shocks and repetitive stimulation at the rate of 1 000 shocks/sec were used Sixteen out of 23 fibres tested in this respect could be excited through indirect electrical stimulation

The uncrossed olivo-cochlear fibres responded with only one impulse to a single shock to the ipsilateral cochlear nerve In 11 fibres, the shortest latencies to single shocks were in the range 3 0—6 0 msec and for a twelfth fibre this latency was 12 msec Responses with latencies about 1 msec implying antidromic stimulation through current spread have been excluded from this series of observations

Repetitive electrical stimulation of the nerve was used on a few occasions In one unit the firing induced by a given sound stimulus had its highest rate when the sound was presented immediately after the end of a repetitive burst of electrical shocks given to the ipsilateral nerve Another unit fired faster when sound and electrical repetitive

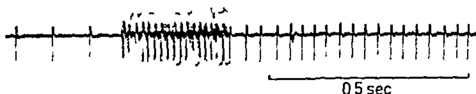


Fig 7 Responses of an uncrossed efferent fibre to repetitive electrical stimulation of the ipsilateral cochlear nerve Note the low regular firing rate with no initial burst and also the increase of firing rate above the resting level which followed the stimulation

stimuli were presented simultaneously than when either stimulation was presented alone In this experiment several of the other uncrossed efferents were stimulated antidromically simultaneously with the orthodromic stimulation of the afferents

An increase of firing rate was seen in 2 uncrossed olivo-cochlear fibres during repetitive electrical stimulation of the ipsilateral cochlear nerve (Fig 7) The firing rate was low and regular similar to that induced by sound stimuli In the unit of Fig 7 and in two other units a long lasting increase of the firing rate followed repetitive electrical stimulation In 3 units with no resting activity such stimulation induced regular after discharges which in one unit reached a firing rate of 40 imp sec

No certain inhibition could be induced through electrical stimulation in any of the 6 units with resting activity studied in this respect Two of these units were tested for inhibition by sound one showed such inhibition

Discussion

The recording conditions when the cochlear nerve was stimulated 0 5 mm from the recording site in the vestibulo-cochlear anastomosis were exactly the same as in a previous work (Fex 1962) It was concluded (Fex 1962) that current spread an artificial synapse Granit and Skoglund 1945 a and b) or some kind of ephaptic transmission cf Lloyd 1942) could not explain the occurrence of spikes in crossed olivo-cochlear fibres in the anastomosis which were driven with a latency of 3 5 msec or more The conclusion can be extended to cover the responses of fibres with latencies down to 3 0 msec in the present work These responses are thus (see below ad 4 and ad 5) evidence of efferent activity

Fibres in the vestibulo-cochlear anastomosis may be 1) unmyelinated autonomic fibres 2 efferent fibres from the reticular system of the medulla (Rossi and Cortesina 1962 3 crossed olivo-cochlear fibres Rasmussen 1946 1953 1960) 4 uncrossed olivo-cochlear fibres Rasmussen 1960 and 5 primary auditory fibres (Rasmussen 1953

ad 1 Unmyelinated autonomic fibres have neither been found in the anastomosis nor definitely been excluded (a k and Rasmussen 1961 Even if such fibres exist it is most unlikely that they ul l be recorded from b th e techniques used in the present work

ad 2 Uncrossed efferent fibres are claimed by Rossi and Cortesina (19C?) to run from cells at different lev l in the medulla f rming an uncrossed efferent reticular

bundle which joins the crossed olivo-cochlear bundle immediately beside the median raphe close to the floor of the fourth ventricle The further peripheral course has not been analyzed and more specifically the results do not permit any conclusion as to whether or not reticular efferent fibres form part of the vestibulo-cochlear anastomosis However Rasmussen (1953 1960) holds the opinion that olivo-cochlear fibres with the possible addition of a small number of primary auditory afferents fully account for the myelinated fibres in the vestibulo-cochlear anastomosis Even if reticulo-cochlear fibres exist in the vestibulo-cochlear anastomosis this would not be critical for the present results since such fibres would have been divided (see Rossi and Cortesina 1962) by the cut made in the floor of the fourth ventricle (see Methods)

ad 3 The crossed olivo-cochlear fibres were divided just lateral of their crossing in the midline of the floor of the fourth ventricle (Rasmussen 1946) The cut was made extensive to save the necessity of histological controls

ad 4 Of the different possibilities listed above only the uncrossed efferents could have shown activity in the basal fascicles of the vestibulo-cochlear anastomosis since the corresponding part of the organ of Corti had been destroyed The remaining fibres that were influenced through either of the cochlear nerves could only have been uncrossed efferents or primary auditory afferents and it is concluded that they were uncrossed efferents (see below *ad* 5)

ad 5 Deep under the surface of the anastomosis fibres were found with irregular resting activity that could not be influenced by stimulation of either cochlear nerve It could not be determined whether or not these fibres belonged to the anastomosis or were lying underneath it They were considered to be primary auditory afferent fibres This assumption was based on the finding that similar fast irregular resting activity that was not changed by sound stimuli at high sound pressures could be recorded from primary afferents both close to and far from the vestibulo-cochlear anastomosis This activity in its turn had the same characteristics as the resting activity of primary auditory neurons of an intact inner ear (*cf* Tasaki 1954 Katsuki *et al* 1958 Fex 1962 Rupert Moushegian and Galambos 1963) Rupert *et al* (1963 *cf* their Fig 4) described one neuron which differed widely in its characteristics from the other neurons they investigated in the auditory nerve The authors considered this neuron to be most probably an olivo-cochlear fibre It can be added that since it responded to sound with a late phase of inhibition it was presumably an uncrossed efferent

The processes underlying resting activity of primary auditory neurons are as yet little known However the new finding that it can persist when the scalae have been opened suggests strongly that the synaptic mechanisms of the organ of Corti may be functioning even under such conditions and thus that also in such a preparation afferent resting activity could be changed through changes of the outflow of efferent activity In the present experiments all fibres running in the basal fascicles of the vestibulo-cochlear anastomosis were divided before they could have reached their terminals Activity recorded in the basal fascicles and influenced through stimulation of either cochlear nerve could then not have been afferent activity The possibility remains that some activity found in the apical fascicles was afferent Such afferents would have been impinged upon by uncrossed efferents, all other efferents being cut These uncrossed efferents would have been preferentially (*cf* Table I) influenced by low frequency sound through the contralateral ear and or they would have been influenced through electrical stimulation of the ipsilateral nerve Thus the hypothesis that some of the activity in the apical fascicles that was influenced by stimulation of either cochlear

nerve was recorded from afferents would lead to the same main conclusions concerning the uncrossed efferents as the conclusions actually drawn in the present work. However the hypothesis that there are primary auditory fibres running in the vestibulo-cochlear anastomosis which have characteristics quite different from those of other cochlear afferents has no documented experimental evidence to substantiate it and may therefore be rejected. It is concluded that the fibres recorded from in the vestibulo-cochlear anastomosis actually are uncrossed olivo-cochlear fibres whether the fibre activity was evoked by sound stimulation of the contralateral ear or by electrical stimulation of the ipsilateral cochlear nerve.

Activity of the uncrossed cochlear efferents. The uncrossed efferents of these experiments had a regular and low firing rate in contrast with the primary and secondary auditory neurons. This and the long latencies indicate that the input to the uncrossed efferents would be integrated over a considerable time. The same conclusion and for the same reason has already been drawn for the crossed efferents (Fex 1962).

The findings that the uncrossed olivo-cochlear fibres do respond to sound stimulation of the contralateral ear together with Rasmussen's (1960) report that these fibres do not receive connexions from the contralateral cochlear nucleus imply that this stimulation is mediated through auditory neurons of the third or higher order.

The results do not permit any conclusion as to whether the ipsilateral or the contralateral ear is the most effective in activating the uncrossed efferents. The method for finding the neurons introduced a bias: the stimulating electrodes in the brain stem may have interrupted preferentially one of several inputs to the uncrossed neurons and only the apical part of the ipsilateral cochlear nerve was stimulated electrically. However electrical stimulation and sound stimulation of cochlear nerves are not strictly comparable. Electrical stimulation may well give rise to an unphysiological mixture of excitatory and inhibitory influences. The shorter latency obtained in response to electrical stimulation may be explained by the shortened pathway. Concerning the crossed olivo-cochlear fibres results on cats with both ears intact suggested strongly that these fibres are more effectively activated through the ear to which they run than through the other ear (Fex 1963).

When activated by sound stimulation of the contralateral ear uncrossed olivo-cochlear fibres in the basal fascicle of the vestibulo-cochlear anastomosis preferentially responded to higher tone frequencies than did the fibres in the apical fascicle. In other words activity in afferent endings in the basal (apical) part of the one cochlea gives rise to activity in the terminals of the uncrossed efferents in the basal (apical) part of the opposite cochlea. This combined with the assumption that the uncrossed olivo-cochlear fibres end in the organ of Corti like the crossed efferents (Iurato 1962, Kimura and Wersäll 1962, Smith and Rasmussen 1963, Spoendlin and Gacek 1963) leads to the hypothesis that the one cochlea is projected upon the other so that afferents and uncrossed efferents from homotopic cochlear points in opposite cochleae are connected. This general principle has already been formulated for the crossed efferents (Fex 1962). The selective depletion of acetylcholinesterase in the cochlea in the basal part after stimulation with high tones in the apical part after low tones (Vinnikov and Titova 1958, Conti 1961) also points to an orderly arrangement of efferent nerve endings.

Because so few results were obtained it cannot be decided whether or not electrical stimulation of the apical part of the ipsilateral cochlear nerve preferentially excited apically directed uncrossed olivo-cochlear fibres.

Uncrossed olivo-cochlear fibres were found in all parts of the vestibulo-cochlear anastomosis implying that they may influence the activity in the organ of Corti in all parts of the cochlea To do this these fibres must ramify extensively since they number only about 125 (Rasmussen 1960) A sign of such an extensive ramification may be seen in the wide range of tone frequencies over which many of the uncrossed efferents respond at the excitatory threshold (*cf* Fig 4) as well as at high sound pressures (*cf* Table I)

In showing inhibition the uncrossed differed significantly from the crossed efferents Inhibition of resting activity by sound stimulation of the contralateral ear was found in all but five out of 44 uncrossed efferents tested in this respect The brain stem electrodes may have interfered with the inputs to four out of these five units The results therefore permit the conclusion that sound stimulation of the contralateral ear as a rule inhibits the resting activity of uncrossed olivo-cochlear efferents with few exceptions The possibility that stimulation of the contralateral ear given a proper choice of tone frequency can inhibit activity in all uncrossed olivo-cochlear fibres must still be left open

Although few the figures of Table I for best inhibitory frequencies together with the findings illustrated in Fig 5 suggest that inhibitory influences may sharpen up the hypothesized orderly arrangement of the uncrossed efferents through which each cochlea is projected upon the opposite cochlea At high sound pressures the unit of Fig 5 was highly sensitive to small changes of tone frequencies at a certain critical frequency It may well be that without the inhibitory influences at play the excitatory sound pressure curve of this unit would have shown the same slope or lack of high frequency cut-off as did the curves of many of the other units

It seems probable that inhibition by sound at a sub-collicular or collicular level of uncrossed efferent cochlear activity can be of great functional importance constituting a means through which the sum of uncrossed and crossed efferent output to the organ of Corti can be better or more finely controlled than if excitatory influences only were at play

Function of the uncrossed cochlear efferents The present new findings that the activity of uncrossed olivo-cochlear fibres can be influenced by both cochlear nerves permit the conclusion that these efferents form part of a feedback system within the auditory system as do the crossed olivo-cochlear fibres (Fex 1962) The function of both systems is still unknown but some evidence has been offered that they may have a centrally controlled activity Thus the action potential component (AP) of the round window response to a click decreases slightly during habituation (Altman 1960 Burgeat Andrianjatovo and Burgeat Menguy 1963 Vesely 1963) and during the development of an orientation reaction (Marusева 1961) All findings could be obtained in animals in which the middle ear muscles had been put out of function and so were interpreted as true centrifugal effects The effect of the habituation on the AP was inhibited through cortical ablation (Burgeat Andrianjatovo and Burgeat Menguy 1963) or through spreading cortical depression (Vesely 1963) Unfortunately all these studies lacked controls with the cochlear efferents cut and are thus not finally conclusive

To the observation that the above mentioned changes of the AP were small the following should be added It was found in a macroelectrode study (Desmedt and LaGrutta 1963) that the effect of electrical stimulation of the uncrossed olivo-cochlear fibres is small at low rates of stimulation The same holds true for the crossed cochlear efferents (Galambos 1956 In the present work it has been demonstrated that the

firing rate of uncrossed cochlear efferents is low and so is the firing rate of the crossed efferents (Fex 1962) The conclusion is that if the firing rate of the efferents as found in these experiments on decerebrate cats is representative then the effect of the efferents on cochlear events under physiological conditions must be small as expressed in db This conclusion is of course fully compatible with the assumption that the cochlear efferents are of great functional importance

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Identification and Cellular Localization of the Catecholamines in the Retina and the Choroid of the Rabbit

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Abstract

Häggendal, J. and T. Malmfors. *Identification and cellular localization of the catecholamines in the retina and the choroid of the rabbit.* Acta physiol. scand. 1965. 64. 58—66. — By means of fluorescence microscopical and chemical methods the catecholamines of retinas and choroids were examined in rabbits normally, after treatment with drugs interfering with catecholamine storage and after bilateral cervical sympathectomy. Dopamine was found to be the dominating catecholamine in the retina (0.1—0.2 µg per g). It was localized to particular neurons. The results support the view that dopamine serves as the transmittor of these neurons. Noradrenaline was found in the choroid (0.[illegible]—0.3 µg per g). It was localized to adrenergic vascular nerves.

Noradrenaline (NA) and acetylcholine are generally recognized as transmitters in the peripheral nervous system. They have also been found in the central nervous system (CNS) and have been suggested to exert the same function there. Other compounds have also been discussed as transmitter substances in the CNS, for instance dopamine (DA) and 5-hydroxytryptamine (5-HT). Recently conclusive evidence has accumulated in support of the view that monoamines serve as transmitters in the CNS. *First*, NA, DA and 5-HT have been demonstrated in central neurons by means of a histochemical technique, the intraneuronal distribution being essentially the same as that of NA in peripheral adrenergic neurons (Carlsson *et al.* 1962, Carlsson, Falck and Hillarp 1962, Dahlström and Fuxe 1964 a and b, Fuxe 1964, Carlsson *et al.* 1964). *Second*, the disappearance of monoamines and one of the enzymes responsible for their synthesis following axotomy has been demonstrated (Magnusson and Rosengren 1963, Carlsson, Magnusson and Rosengren 1963, Andén *et al.* 1964 a, b and c, Dahlström and Fuxe 1964 c). *Third*, the release of a monoamine following stimulation of central axons has been observed (Andén *et al.* 1964 d).

The retina is considered to be a part of the central nervous system from embryological, histological and functional points of view.

Nobody seems to have demonstrated any of the above mentioned compounds in the retina. Specific cholinesterase has been found by histochemical methods in the retina of the cat suggesting that acetylcholine may serve as a transmitter in the retina (Koelle *et al.* 1952).

Dunér, Euler and Pernow (1954) found the NA content of the choroid in the cow to be 0.3—0.4 μg per g wet weight. In the retina measurable quantities of NA or adrenaline (A) were not found ($<$ 0.05 and $<$ 0.005 μg/g, respectively). Using a fluorimetric method Bernheimer (1964) was not able to demonstrate any catecholamines in the retina of calf ($<$ 0.01 μg/g).

Reserpine treated animals show pronounced sensitivity to light and extreme miosis. Reserpine causes depletion of NA in the adrenergic nerve tissues which then cease to function (Carlsson *et al.* 1957). Since the cause of the increased light sensitivity might be a blockade of an aminergic inhibitory transmission mechanism, aminergic neurons in the retina were looked for.

When the fluorescence microscopical method of Falck *et al.* (1962) was applied to the retina of untreated rats, fluorescent nerve cell bodies and cell processes were discovered. From pharmacological experiments and histochemical tests there was good reason to believe that the fluorescence was derived from a primary catecholamine such as DA or NA (Malmfors 1963). In order to establish the identity of this amine it was found necessary to use a sensitive chemical method like that of Häggendal (1963).

The discovered neuron system is much more abundant in the retina of rabbits than in the retinas from several other examined animals.

In a preliminary report it was found that DA was the dominant catecholamine in the retina of rabbits, but a small amount of NA was also detected (Häggendal and Malmfors 1963).

The present paper gives detailed data concerning the occurrence and localization of catecholamines in the retina and, for comparison, in the choroid of rabbits normally, after cervical sympathectomy and after treatment with drugs.

Material and methods

About 100 mixed albino and pigmented rabbits of both sexes with a body weight of 2—3 kg were used. Different groups of animals were treated as follows:

a) Reserpine 2.5 mg per kg i.v. 16 hrs before the preparation.
b) Two doses of 200 mg α-methyl-meta-tyrosine (α-MMT) per kg i.v. with an interval of 2 hrs. The first injection was given 24, 48 or 96 hrs before the preparation.
c) For histochemical purpose rabbits were treated with the same doses of α-MMT also 16 and 72 hrs before death. Other animals were treated with 3 doses of 100 mg meta-tyrosine per kg i.v. with intervals of 2 hrs. They were killed 3 hrs after the last dose. Still other rabbits were treated with 100 mg per kg nialamide i.p. 5 hrs before the preparation.
d) Bilateral cervical sympathectomy was performed under ether anaesthesia 8 days before the preparation.

The animals were killed by air-embolism. The eyes were immediately dissected out and divided about 2 mm behind the limbus. The preparation for the biochemical determinations was started by making a cut in the posterior half of the eye ball. The retina was carefully prepared free from the underlying choroid. Some of the pigment epithelium of the retina followed the retina, but part of it remained on the choroid. Then the retina was cut free from the optic papilla and placed in ice-cold 0.4 perchloric acid. Tissues from at least 3 animals were pooled for every separate determination of both the retina and the choroid.

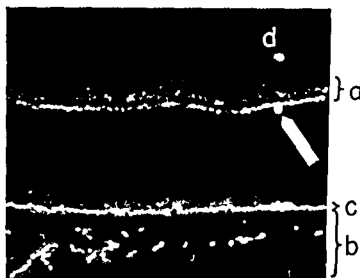


Fig. 1. Retina and choroid of a rabbit treated with nialamide. In the inner nuclear layer of the retina one fluorescent cell body is found (arrow). In the inner plexiform layer (a) three separate layers of fluorescent varicose fibers are seen. In the choroid (b) fluorescent adrenergic nerves are distributed around the vessels. The pigment epithelium (c) shows a high autofluorescence. (d) is an artifact. Fluorescence photomicrograph. Magnification 210.

The samples were homogenized and centrifuged. For the determination of the catechol amines the pH of the extracts was adjusted to . . The extracts were then allowed to pass through a ion-exchange column Dowex 50 W X8 with a length of 35 mm and a diameter of 2.5 mm. After washing with water and buffer the elution was at first performed with N hydrochloric acid in three 1 ml fractions and then continued with 2N hydrochloric acid in . 8 1 ml fractions (for details see Häggendal 1963). The first 3 fractions were estimated for NA and A according to Häggendal (1963) and the other fractions for DA according to Carlsson and Waldeck (1958) modified by Carlsson and Lindqvist (1962). The fluorescence was read in an Aminco-Bowman spectrophotofluorometer and the activating and fluorescence spectra were recorded from every fraction and compared with standard solutions of synthetic NA, A and DA. The determination of 5-HT was made according to Bertler (1961).

For the histochemical study small pieces . mm were cut out from different parts of the posterior half of the eye ball and treated essentially according to Falck (1962). The pieces were freeze-dried and treated with formaldehyde gas of optimum humidity (Hamberger, Malmfors and Sachs, to be published), embedded in paraffin, sectioned and mounted. The sections were then examined and some of them photographed in a fluorescence microscope with a dark field condenser and a Kodak Wratten filter . in the tube. The source of the activation light was an Osram HBO 200 high pressure mercury lamp with a 3 or 4 mm Schott BG 12 filter.

Results

Histochemical findings

In the sections from the untreated animals a strong yellow-green fluorescence was found. The specific fluorescence in the retina was located to cell bodies in the inner part of the inner nuclear layer and to very fine cell processes with stronger fluorescent varicosities in the inner plexiform layer. The fluorescent structures in the latter layer were accumulated in three separate layers parallel with one another and with the internal

TABLE I Schematic illustration of the fluorescence as revealed by the histochemical method

	Normal	Res treat	M tyrosine	α MMT 16 h	α MMT 24 h	MMT 72 h	Denerv	Nial treat
Retina	+++	—	+	++	+++	+++	+++	+ - - -
Choroid	+++	—	+	+	+	+++	—	+++-

TABLE II Catecholamines in the retina and choroid of the rabbit

Each determination as performed on 6—10 eyes		ng per retina DA	ng per choroid NA	µg per g wet weight retina DA	µg per g wet weight choroid NA
Normal		3 4	13 6	0 04	0 22
		6 0	5 3	0 07	0 11
		9 5	22 7	0 14	0 43
		7 7	15 0	0 12	0 29
Mean values		5 3	14 1	0 09	0 26
Reserpine treated		0 2	0 2	0 00	0 00
		0 9	—	0 01	—
		2 4	0 0	0 04	0 00
Mean values		1 2	0 1	0 02	0 00
α MM treated	After 24 h	—	0 0	—	0 00
	After 24 h	2 6	—	0 04	—
	After 48 h	6 2	7 4	0 13	0 31
	After 96 h	7 2	10 7	0 12	0 20
Sympathectomized	After 8 days	9 8	0 0	0 09	0 00
		4 6	0 0	0 05	0 00
		11 5	—	0 13	—
		3 1	0 1	0 04	0 00
		8 8	—	0 20	—
Mean values		7 6	0 0	0 10	0 00

limiting membrane (Fig 1) The fine varicose fibers had the same appearance as synaptic terminals revealed by the same technique in the hypothalamus and other NA rich parts of the CNS Carlsson *et al* 1962 Carlsson Falck and Hillarp 1962 Fuxe 1964 The outer layer of the fluorescent fibers which showed the most pronounced

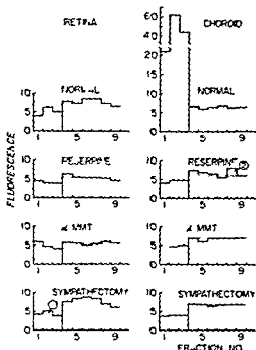


Fig 2 Separation of noradrenaline (NA) and dopamine (DA) in extracts of retina and choroid on a strong cat on exchange resin column
Column Dowex 50 W—X8 length 3 mm diameter 2 7 mm Na form
Elution the first 3 ml with N HCl the rest with 2 N HCl in 1 ml fractions The first 3 fractions were assayed for NA and the rest of the fractions for DA according to the principles of the hydroxy indole method The figures show the mean values of the fluorescence intensities of the fractions The figures correspond to Table II Dotted lines indicate the blanks for NA and DA respectively
Spectra not typical for NA
Spectra not typical for DA

fluorescence was situated just at the border between the inner nuclear layer and the inner plexiform layer and the fibers seemed to surround non fluorescent cell bodies The second layer was situated in the middle of the inner plexiform layer and none of its fibers seemed to be in contact with a non fluorescent cell body The third inner layer which was the weakest was situated quite near the ganglion cell layer and some of the fibers seemed to be in contact with a few of the ganglion cells Some very weakly fluorescent cell processes were found between the three layers but none in the ganglion cell layer in the nerve fiber layer or outside the inner part of the inner nuclear layer

Some of the specimens from untreated rabbits were treated with formaldehyde gas for 3 hrs at 80 C The fluorescence in the sections from these specimens was about the same as in the sections from specimens treated for only 1 hr Thus no support for the presence of a secondary catecholamine was obtained

In the choroid specific fluorescence was found to be localized to fibers with very strongly fluorescent varicosities. The fibers displayed the same appearance as adrenergic NA-containing nerve fibers in other organs treated in the same way The fluorescent nerve fibers were situated close to the outside of the media of the large vessels especially the arterioles No adrenergic nerve fibers were demonstrated around the retinal vessels except in the optic papilla and these nerve fibers did not seem to accompany the vessels out over the retinal surface

In the sections from the animals treated with reserpine specific fluorescence was found neither in the retina nor in the choroid except very small faint rests in some cell bodies The background fluorescence was unchanged

In the sections from animals treated with meta tyrosine the specific fluorescence was greatly reduced but some weak fluorescence remained both in the cell bodies and the

TABLE III Catecholamines in the ret na and choroid of the normal rabbit

	Retina		Choroid		
Normal values	NA	DA	NA	DA	
The mean values of table II 6—10 eyes n every estimation	0 07 0 00	5 3 0 09	14 1 0 2o	2 9 0 06	ng per eye μg per g wet weight re covery of 50 ng NA and DA added to tissue extract about 30 per cent
Results of one est mation on 20 eyes	0 13 0 00	12 5 0 19	8 0 0 17	2 6 0 0o	ng per eye μg per g wet weight re covery of 50 ng NA and DA added to tissue extract about 100 per cent
Results from earl er ex periments (Haggendal Malmfors 1963)					
8 eyes	0 5	13	—		ng per eye
26 eyes	0 0o	8	10	0	

cell processes in the retina as well as in the nerve fibers in the choroid The back-ground fluorescence had increased presumably due to accumulation of meta tyrosine in the tissues

In the sections from the animals treated with α MMT the fluorescence in the retina seemed to be slightly reduced 16 hrs after the treatment while it seemed to have returned to almost the same intensity as in those from untreated animals 24 and 72 hrs after the treatment The specific fluorescence in the choroid was markedly reduced 16 and 24 hrs after the α MMT treatment but was almost the same as that of the normal animals 72 hrs after the treatment The back ground fluorescence was weaker than that of the meta tyrosine treated animals and about the same as in the untreated animals Eight days after the bilateral excision of the cervical sympathetic chain the fluorescence in the retina seemed to be unchanged while there were no fluorescent nerve fibers in the choroid

The distribution of the fluorescence in the nialamide-treated animals seemed to be the same as in the untreated animals but the intensity was increased both in the retina and in the choroid

A schematic illustration of the results with the histochemical methods are given in Table I

Biochem cal find ngs

In some of the retina samples of the untreated rabbits activating and fluorescent spectra typical of NA were found in fraction 2 No A was found DA was regularly detected in the retina samples (Table II) The fluorescence intensities of the fractions referring to

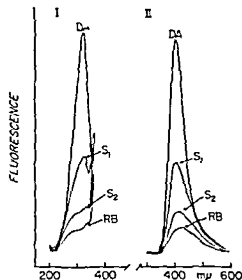


Fig 3

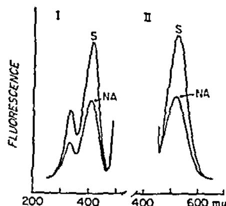


Fig 4

Fig 3 (Left) Activation and fluorescence spectra of eluate from retina extract of 20 rabbit eyes
I Activation spectrum Fluorescent wavelength 400 mμ
II Fluorescence spectrum Activating wavelength 3 0 mμ
DA Dopamine standard RB reagent blank
S sample corresponding to fraction no 6 in Fig 2
S sample corresponding to fraction no 9

Fig 4 Right Activation and fluorescence spectra of eluate from choroid of 20 rabbit eyes
I Activation spectrum Fluorescent wavelength 515 mμ
II Fluorescence spectrum Activating wavelength 400 mμ
NA Noradrenaline standard
S sample fraction no

DA fractions 4—10 were highest in fractions 5—7 Fig 2 The intensities were very small but activating and fluorescence spectra typical for DA were recorded (Fig 3 In the determinations where 50 ng DA had been added to the retinal extract the highest fluorescence intensities appeared in fractions 5—7 The recovery of the added amount of DA was about 100 per cent in the experiment where the estimation was performed on 20 eyes Table III *In other experiments however the recovery was only* about 50 per cent

In the choroid of the untreated animals fraction 2 had activating and fluorescence spectra typical of NA Fig 4 No A was found in this fraction The fluorescence intensities in the fractions referring to DA fractions 4—10 were lower than those from the retina

No signs of 5-HT were found in the activating and fluorescence spectra from the retina and the choroid preparations of untreated animals

After reserpine treatment the DA values in the retina and the NA values in the choroid were markedly reduced

In the retinas and the choroids of the α MMT treated animals the catecholamines were found in very small amounts 4 hr after the treatment *The amounts of NA in the*

choroid and the amounts of DA in the retina were almost the same as those in the untreated animals 48 and 96 hrs after the same treatment

The NA had disappeared in the choroids of the sympathectomized animals However the amounts of DA in the retinas of these animals seemed to be in the normal range

Discussion

The histochemical observations indicate that the specific fluorescence observed in the retina and choroid is derived from primary catecholamines These amines were identified by column chromatography and spectrophotofluorometry With column chromatography synthetic DA added to the extract of retina appeared in the same fractions as the compound from the normal retina of untreated animals Synthetic DA and the compound from the normal retina showed identical activating and fluorescence spectra In the same manner the identity of NA in the choroid was established

The great reduction of the DA in the retina and of the NA in the choroid 16 hrs after a rather high dose of reserpine can be taken as a further support of the identities

The results of the biochemical determinations show that DA is the dominating monoamine in the retina of untreated rabbits The amounts of DA however are very low That DA has not been demonstrated earlier in the retina may be explained by the higher sensitivity of our procedure The variations in our different determinations are presumably due to the great technical difficulties especially to get constant recoveries and blanks The amounts of DA in the retina have been calculated to about 0 1—0 2 μg/g tissue wet weight and 10—15 ng/retina No definite difference has been found between pigmented and albino rabbits

In the choroid of rabbit NA was found in about the same amounts as previously described by Dunér Euler and Pernow (1954) in the cow and by Bernheimer (1964) in the calf Further work is needed to establish the possible presence of small amounts of DA in the choroid

The fluorescence intensity in the histological sections of retina was reduced 16 but not 24 hrs after the α MMT treatment (Table I) Chemically however there was still a reduction of the DA after 24 hrs (Table II) This suggests that a moderate reduction in DA (about 50 per cent) will not be detected by means of the present histochemical technique

The disappearance of the fluorescent fibers and the strong reduction of the NA in the choroid after sympathectomy show that these fibers belong to the sympathetic nervous system In the retina however the DA was not significantly influenced by the sympathetic denervation The fluorescent structures in the retina thus does not appear to be connected with the cervical sympathetic chain

Our results indicate that DA is the dominating catecholamine in the retina of the rabbit and that the DA is localized to fluorescent cell bodies and varicose fibers which *seem to belong to each other The data are consistent with the view that DA serves as* the transmitter of these neurons

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A Comparison of Neural and Psychophysical Responses to Taste Stimuli in Man

By

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Abstract

Diamant H B Oakley L Strom C Wells and Y Zotterman *A comparison of neural and psychophysical responses to taste stimuli in man* Acta physiol scand 1965 64 67—74 — Electrophysiological records of taste activity have been obtained from the chorda tympani nerve of otosclerotic patients When possible preoperative psychophysical responses to taste stimuli were also obtained The summated chorda tympani discharge to 0 2 M NaCl adapts completely and the time required agrees with psychophysical reports Psychophysical estimates of the sweetness of different sugars correspond closely with nerve response magnitudes Gymnema extract abolishes both the sweet sensation and the nerve response to sweet tasting chemicals The total sum of taste activity in the entire chorda tympani nerve appears to be an important determinant of the psychophysical responses There are large individual differences in the relative size of the neural response to different taste stimuli Responses to ethyl alcohol are described

Electrophysiological records from the human chorda tympani nerve can provide information about the relationship between the taste stimulus and the neural response These neural responses in turn may be related to the corresponding psychophysical responses

The choice of the electrophysiological recording technique is particularly important Is the rectified summated and filtered transformation of the activity of the whole chorda tympani nerve related to the intensities and qualities of the taste sensations we experience We do not know whether the central nervous system "sees" the responses from the chorda tympani in the same way as our electronic apparatus What we record might be just an epiphenomenon that parallels in time the true and unrecorded information bearing responses of the peripheral taste nerve However if there is a good

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correspondence between the summated chorda tympani discharge and the psychophysical responses we can infer not only that we have chosen a meaningful recording technique to tap into the peripheral input line but also that the central mechanisms do not seriously alter the input If such a correspondence is lacking we must conclude that either an inappropriate recording technique was used and/or the sensory input is modified in the central nervous system

This report complements previous studies of human electrophysiological taste responses (Diamant and Zotterman 1959 Diamant Skoog and Zotterman 1960 *Diamant et al 1963*) by placing greater emphasis upon the relationship between nerve activity and psychophysical responses Evidence will be presented which indicates that the summated response of the whole chorda tympani nerve is a valid and relevant measure of taste activity

Methods

1 Subjects and materials

These experiments were carried out in the Department of Otorhinolaryngology University of Umeå in January and April of 1964 The data in this study are based upon results from 6 patients designated as follows

Patient	Sex	Age
1	female	26
2	female	40
3	male	54
4	male	33
5	female	50
6	female	35

All chemical solutions were made up in distilled water Tap water was used for warming and cooling the tongue The following chemicals were used sucrose lactose maltose glucose fructose galactose sodium cyclamate sodium saccharin glycerin sodium chloride citric acid *ethanol quinine sulphate quinine (mono)hydrochloride hydrate and phenylthiocarbamide*

Gymnema extract was prepared as a water decoction of dried leaves of *Gymnema sylvestre* These were heated for four hours at 8[illegible] C and the water extract removed In the case of patients no 1 2 3 the final concentration was 0 2 g leaves ml solution and for patients no 4 5 6 it was 0 4 g leaves/ml solution This corresponds to 1 percent and 2 percent solutions of purified Gymnema extract according to Warren and Pfaffmann (19[illegible]9)

2 Neural recording

Special precautions were taken to avoid 50 cps mains interference since the recording had to be performed in an electrically unscreened room The patient was grounded through metal collars around the neck and arm The nerve impulses were recorded via an RC amplifier which incorporated a twin T rejection filter tuned to 50 cps By applying heavy negative feedback around the filter the rejection bandwidth was reduced to about [illegible] cps at 3 db down This *permitted the recording of the response from the chorda tympani in the presence of a 50 cps* disturbance ten times larger than the nerve impulses The amplified response was recorded on one channel of a stereo tape recorder Tandberg while the other channel was used for a voice recording of the protocol *The impulses were monitored on an oscilloscope Later the tape* recorded data was processed with an electronic summator and Grass Polygraph ink writer Prototypes of the summator have been in use for ten years in the *Department of Physiology* and its simple circuit design is shown in Fig 1 With the last patient the surface temperature of the tongue was recorded with a thermocouple and both this and the summator output were displayed on 1 n[illegible] separate channels of a Grass ink writer

The experiments were performed under light general anesthesia (N_2O Fluothane and the patients were *immobilized with succinylcholine* to prevent movement artifacts The chorda tympani nerve was visualized through middle ear cut naturally and the sheath removed The

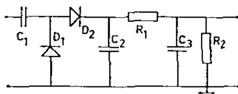


Fig 1 Schemat c diagram of the summator (integrator) circuit Th s is preceded by a oltage ampl ficat on stage (EF86) with ga n control
$C_1 = 10\mu F$ C = C = 0 1 — 10 F stepwise variable D = D = OA81 R = 1 M Ω R ≈ 1 M Ω
R may be part of the recording dev ce

nerve activity was recorded th ough a ingle platinum electrode and the speculum served as the reference electrode A special plastic stimulus dispenser was used Its tip was located at the tongue surface and the 15 ml of taste solut on ere held in the dispenser reservoir some 25 cm above the tongue All taste solutions were warmed to approximately 30 C The recording sessions lasted for about 1 hr

3 Psychophysical test

Various psychophysical tests were carried out on four pati nts the day before their operat on Only with patients no 3 and 4 did we fully succeed in obtaining both psychophys cal and nerve responses All taste solutions were at room temperature and distilled wate was used as the r nse between the stimulations The following psychophysical tests were carried out

A Time for the complete adaptation of the salt taste The patient held about 10 ml of 0.2 M NaCl n his mouth He was instructed not to move his tongue vigo ously and to raise his ha d when the salt taste disappeared

B The patients were given 0 005 M phenylthiocarbam de and asked to report what they tasted Th s was a quick test for taste blindness

C Magnitude estimation of the sweetness of sugars The following solutions were tasted once in forward and once in reverse order 0.5 M solutions of sucrose fructose glucose galactose lactose and maltose 0 004 M sod um saccharin 0 03 M sodium cyclamate and 0 5 M sucrose The pat ent was a ked to judge the sweetness of each solution and compare it w th the standard 0 5 M sucrose wh ch was given a sweetness rating of 100 If a solution tasted one half as sweet he was to call it 50 etc Pat ents no 4 5 6 received the standard before each of the comparisons Patient no 3 rece ved the standa d only at the beginning and end of the two runs

D The effect of Gymnema extract upon taste The sequence of taste solutions was 0 2 M NaCl 0 5 M sucrose 0 0? M citric acid 0 005 M quinine hydrochloride (or 0 003 M qu nine sulphate) — Gymnema extract applied to the tongue for 2 min — 0.2 M NaCl 0 5 M sucrose 0 02 M citric acid 0 005 M quinine hydrochloride (or 0 003 M qu nine sulphate) 0 5 M sucrose The patient was instructed to judge only the strength of each taste solution calling 0.2 M N Cl a strength of 100 He was told to ignore the qual ty of the solutions After the Gymnema ex ract treatment he was aga n told to call the intens ty of the salt 100

A cotton Q tip appl cator was used to apply the solut ons to the tongue of patient no 3 in test C and D S nce a diseased ear can produce abnormal taste function the logic of this testing procedure wa to restrict the solution to the anterio part and psilateral s de of the tongue Ho ever the esults f om nerve record ng suggested that more reliable psychophysi l procedures might impro e the corresponden e between psy hophysical and neural results For this eason pat ents no 4 5 and 6 were instructed to take about 5 ml in the front of the mouth and taste normally

Results

The summated chorda tympani nerve response to 0 2 M NaCl is show n in Fig 2 The decline in nerve activity to 3 min of salt stimulation for each of 3 patients may be seen in records A B C During this 3 min period there was a continuous flo v of salt over the

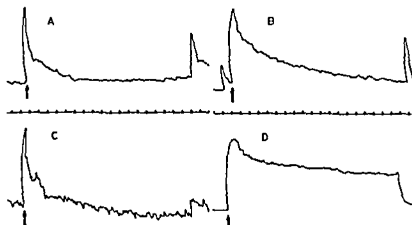


Fig. 2 The summated chorda tympani response to a continuous 3 min flow of 0.2 M NaCl. A, B, C are human responses for patients no. 1, 2, 3 respectively and D is a rat response. Dots indicate response during application of distilled water, arrows onset of salt. Tape recorded data processed under identical conditions with rise and fall time constants of 1.5 sec. The tape recorder was off at beginning of B. Time base in 10 sec intervals.

tongue at a rate of 7 ml/sec. The initial large response to salt is indicated by an arrow while the application of distilled water is indicated by a dot above the curves. (The responses to water here were due primarily if not wholly to cooling and/or mechanical stimulation.) Record A is the most satisfactory from the technical point of view, for the signal-to-noise ratio was good and the baseline quite stable. The response was 95 percent adapted within 50 sec. In addition to the 3 records shown here the adaptation of the salt response of patients no. 4 and 6 was very similar to that shown in A and C. Our records of human neural adaptation to sodium chloride distinctly contrast with record D which is taken from the rat chorda tympani. Here the response declines very slowly over a 3 min period, and when the record is continued for some minutes more little or no further decline in amplitude is seen (Beidler 1953, Zotterman 1956). Patient no. 3, whose neural response is shown in record C, indicated in the psychophysical test *that the salt taste disappeared after 90 sec. Patient no. 4 indicated that he could no* longer taste salt after 79 sec, which corresponded to a 95 per cent reduction in the magnitude of his neural response.

Some individuals are "taste blind" for phenylthiocarbamide (Hoffmann 1959) and we attempted to determine whether this deficiency has a peripheral basis. However the 3 patients tested (no. 4, 5, 6) all reported an intense bitter taste.

There are rather substantial individual differences in taste sensitivity to sweet chemicals (Cameron 1947). For this reason it is of relatively little value to compare the nerve responses of individual patients with psychophysical responses from other subjects. We were able with [illegible] patients to obtain both psychophysical magnitude estimates of the relative sweetness of different chemicals and summated chorda tympani discharges to these same chemicals (Table I). The values in each column have been rounded to the nearest 5 per cent and are relative to the response to 0.5 M sucrose, which has been set at 100. The psychophysical reports are means of [illegible] determinations while the neural

TABLE I Comparison of psychophysical and neural response to sweet tasting substances The values in each column are relative to 0.5 M sucrose set at 100 The maximum height of the summator record was measured

Stimulus	Patient 3		Patient 4	
	Psy	Neur	Psy	Neur
0.5 M sucrose	100	100	100	100
0.5 M fructose	100	100	80	80
0.5 M maltose	—	40	75	60
0.5 M galactose	40	45	45	40
0.5 M lactose	45	45	30	30
0.5 M glucose	25	45	35	40
0.004 M Na saccharin	100	65	125	105
0.03 M Na cyclamate	55	80	115	100

TABLE II Individual differences in the chorda tympani discharge. The values in each column are relative to 0.5 M sucrose set at 100 The maximum height of the summator record was measured

Stimulus	Patient no			
	1	2	3	4
0.5 M sucrose	100	100	100	100
0.2 M NaCl	105	100	150	50
0.02 M citric acid	155	195	60	100
0.004 M Na saccharin	50	70	65	105
0.03 M Na cyclamate	90	120	80	100

values are based upon a single determination There is a good correspondence between the psychophysical and neural data especially for the sugars The artificial sweeteners saccharin and cyclamate have qualitatively different tastes from the sugars and this *may have affected the judgments of sweetness The psychophysical method used with* patient no 4 produced the better agreement

We have also found that there is significant individual variation in the response of the chorda tympani to different chemicals A comparison of the neural responses of patients no 3 and 4 in Table I will give an indication of the individual variation for the sugars and artificial sweeteners Diamant *et al* (1963) with a previous patient obtained still another ordering of neural responses to sugars Table II presents individual differences for other chemicals All of the responses are relative to 0.5 M sucrose which has been

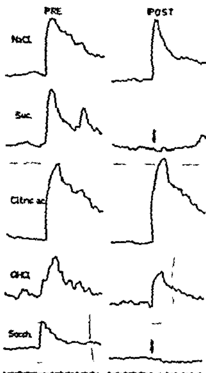


Fig 3 Elimination of the neural response to sweeteners by Gymnema extract The stimuli were presented in the order shown before and after treatment of the tongue for 90 sec with 1 percent Gymnema extract The arrows show the *time at which saccharin and sucrose were applied* The dot in the sucrose record indicates a temperature response to cool distilled water *Time marks* in sec The responses to quinine are displayed at a somewhat higher amplification The pre response to saccharin was *recorded* earlier in the experiment The concentrations were 0.? M NaCl 0.5 M sucrose 0.0? M citric acid 0.00? M quinine hydrochloride and 0.004 M sodium saccharin Data from patient no 1

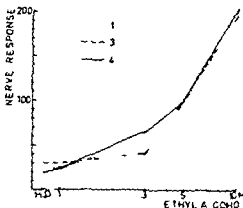


Fig 4 *The* maximum amplitude of the summated chorda tympani response as a function of ethyl alcohol concentration for 3 patients The ordinate gives the relative nerve response where the response of each patient to 5.0 M alcohol has been set at 100

assigned a value of 100 It is readily apparent that there is wide variation in responsiveness to the different chemicals For example patient no 3 had a poor response to 0.? M NaCl and patient n had a very good response to 0.02 M citric acid

Fig 3 shows that following treatment of the tongue with Gymnema extract sucrose and saccharin failed to elicit detectable neural activity We tested 5 patients in this fashion and found that only the neural response to sweet tasting chemicals was

affected viz sucrose fructose saccharin and cyclamate There were no significant effects (i e greater than a 10 per cent change) upon responses to 0 2 M NaCl 0 02 M citric acid or quinine (0 002 M and 0 005 M quinine hydrochloride or 0 003 M quinine sulphate) The basic result in the psychophysical portion of the experiment was the same namely that the sweet taste was eliminated (sucrose) No subjects reported a decrease in bitter taste (quinine)

With ethyl alcohol as a taste stimulus it was necessary to use concentrations in excess of 1 0 M to elicit good nerve responses (Fig 4) These neural responses were positively accelerating functions of log molar concentration as opposed to the usual linear trend with other taste solutions (Diamant *et al* 1963) Use of the strong 5 M and 10 M solutions in 5 out of 6 cases resulted in a slowly rising negatively accelerated summated response which took 10 or 20 sec to reach its maximum height. Concentrations greater than 3 M produce a smarting or burning sensation (Diamant *et al* 1963)

Discussion

Bujas (1953) has studied psychophysical salt adaptation in two subjects Using 0 15 M NaCl to stimulate a tongue area 1 cm in diameter he found complete adaptation in 50 and 54 sec These values are of the same order of magnitude as those given by our patients in psychophysical tests when the whole tongue was stimulated with 0 2 M NaCl (79 90 and 122 sec) The shape of Bujas s psychophysical adaptation curves to 0 15 M NaCl are very similar in form to our neural adaptation records in Fig 2 but there are too many unknown factors (e g the linearity of the ordinates the appropriate psychophysical baseline level) to permit a precise and quantitative comparison of the two sets of curves

Our records of neural adaptation to sodium chloride suggest that adaptation is complete i e the activity decreases until it reaches the resting level of activity In addition there is a reasonable correspondence between the neural and psychophysical records for the time necessary for complete adaptation Thus we may conclude that the human psychophysical observation of rapid and complete salt adaptation can be accounted for by diminished activity in the chorda tympani nerve There is no need to postulate the existence of central adaptation mechanisms

We made no attempt in this series of experiments to split the corda tympani into small strands in order to record from single fibres for previously such efforts failed (Diamant *et al* 1963) As a result we have no direct evidence on the quality specificity of single taste fibres One might argue that within the chorda tympani there is a subset of fibres which are responsive only to sweet tasting solutions and that it is these and not multi-quality fibres (i e those which respond to more than one class of chemicals) which code sweetness However the correspondence for the sugars in Table I suggests that the magnitude of perceived sweetness is determined by the discharge from all of the active fibres within the nerve This inference is also supported by the results with Gymnema extract which show that total elimination of the sweet taste is paralleled by total elimination of the chorda tympani response Thus it is reasonable to conclude that the magnitude of sweetness is determined by all of the activity which is generated in the taste nerves

The summated response however may be too simple a measure to correlate with psychophysical intensity judgments across different taste qualities There may be other

complicating factors such as the temporal discharge pattern or the ultimate central projections

Extracts of *Gymnema sylvestre* when applied to the tongue will temporarily eliminate the ability to taste sweet substances (*e g* Shore 1892) Andersson *et al* (1950) working on dogs and Hagstrom (1957) using hamsters demonstrated that this drug will suppress the chorda tympani response to sucrose while leaving the response to sodium chloride unaffected It is not known whether the behavioural preference for sucrose would also be suppressed or eliminated In the present report we have shown that the same Gymnema extract treatment will eliminate both the neural and psychophysical responses to sweet tasting chemicals in the same human subject Shore also reported that Gymnema extracts eliminated sensitivity to bitter substances In our hands such treatment has had no clear effect on sensitivity to suprathreshold concentrations of quinine either in the psychophysical tests and neural recording sessions with the patients or in psychophysical tests with 3 other normal subjects We are not able to explain this discrepancy

The agreement between the neural and psychophysical data for salt adaptation magnitude of sugar responses and suppression of sweet sensitivity by Gymnema extract indicates that the measurement of the total activity in the nerve without reference to fibre types or ultimate anatomical dispersion in the central nervous system can give relevant and meaningful information about taste activity

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The Distribution of Adrenergic Nerve Fibres to the Blood Vessels in Skeletal Muscle

By

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Abstract

Fuxe K and G Sedvall *The distribution of adrenergic nerve fibres to the blood vessels in skeletal muscle* Acta physiol scand 1965 64 75—86 The distribution and morphological construction of the adrenergic innervation to the various sections of the vascular bed in skeletal muscle of the cat were studied using a specific histochemical fluorescence technique A typical adrenergic ground plexus (Hillarp 1946 1959) was seen directly superimposed on the smooth muscle layer of the vessels The nerve terminals seldom penetrated into this layer The arteries entering the muscles showed only a sparse amount of adrenergic fibres whereas the intra muscular arteries arterioles and metarterioles exhibited a fairly rich supply The innervation was uniform no definite accumulation of adrenergic fibres being observed in any section of the vascular bed The capillaries did not seem to receive any adrenergic fibres The vessels on the venous side had a very sparse innervation Single nerve bundles could be observed in relation to some small venules (20—40 μ) but only occasionally were adrenergic fibres seen around intra muscular veins with diameters above 50 μ Possible mechanisms for the mediation of nervous vasoconstrictor stimuli to the various vessel sections are discussed

Our present knowledge of the nervous control of blood vessels is based predominantly on physiological studies. Such investigations have established the important role of vasoconstrictor nerves in the hemodynamic regulation of most vascular regions in the body (see Folkow 1956 In skeletal muscle the changes in arterial resistance trans capillary transport and tissue volume following sympathetic stimulation indicate the distribution of adrenergic vasoconstrictor fibres to different sections of the vascular bed such as arterioles precapillary sphincters and venules (Folkow and Löfving 1948 Bucherl and Schwab 1953 Folkow and Mellander 1960 Mellander 1960 Renkin and Rosell 1962

The morphological organization of the structures transmitting vasoconstrictor impulses to the smooth muscle cells in the blood vessels has until recently been largely unknown It has moreover been difficult to establish whether or not there are quantita



Fig. 1. *M. gastrocnemius* of cat. Cross-section. Cross-sectioned bundles of adrenergic fibres are present close to the external muscle layer of one artery (A) and one arteriole (a). The veins (V) receive no adrenergic fibres. The internal elastic membrane of the artery shows non-specific autofluorescence. Fluorescence microphotograph. Magnification 165 ×.

tive differences in the amount of vasoconstrictor fibres reaching the various sections of the vascular bed (Mellander 1960, Abboud and Eckstein 1964). Such problems could possibly be solved by studying the muscle vessels with a specific histological method that selectively demonstrates vasoconstrictor nerves.

A specific histochemical fluorescence technique for the demonstration of catechol amines and 5-hydroxytryptamine in tissues has recently been developed (Falck *et al.* 1962, Carlsson, Falck and Hillarp 1962, Falck 1962). This method permits the visualization of the adrenergic transmitter. Due to their high concentration of noradrenaline the adrenergic nerve terminals can be observed everywhere in the tissues by means of this technique.

In the present study this direct method was used to study the organization and distribution of the vasoconstrictor innervation to the blood vessels in skeletal muscle of the cat. A preliminary report of parts of this study has been published earlier (Fuxe and Sedvall 1964).

Methods

The study was performed on about 0 cats of both sexes weight 0 4.0 kg. All animals were anesthetized with sodium pentobarbital (Nembutal®, Abbott, 3 mg/kg i.p.). Small tissue specimens were taken from the small vessels entering the tibialis anterior and gastrocnemius

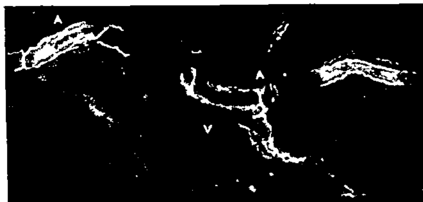


Fig 2 M *gastrocnemius* of cat Longitud nal sect on A network of adrenergic fibres is p esent around a small bifurcating artery (A) The accompanying e n (V) receives no adrenergic fibres Fluorescence microphotograph Magnification 165 ×

muscles S nall intramuscular vessels we e studied in spec mens taken from d fferent parts of the t b al s anterior and gastrocnem us muscles Special care was taken n t to remove the sheath of connective tissue surrounding th larger vessels All specimens were excised in the living animal Immed ately after excision the specimens were fro en in propane cooled by liquid nitrogen After freeze-drying at — 35 C for 5—7 days the tissue specimens were treated with formaldehyde gas at 80 C for 1 hr During this treatment primary catecholamines co idense — without diffus on — with form ldehyde to intensely fluorescent 3 4-dihydroisoquinolines (Corrodi and Hillarp 1963 1964) The specimens were then e nbedded in paraffin (mpt 56—58 C Merck) *in vacuo* Sections were made 10 μ thick and mounted in Entellan® (Merck) after removal of the paraffin (cf Falck 1962 Dahlstrom and Fuxe 1964 a) The sections were examined in light f om an Osram HBO 200 high pressure mercury lamp equipped with a Schott BG 12 filter 4 mm A fluo escence microscope was used w th a dark field condensor (Ze ss) and a Zeiss 50 absorption filter (2 mm) in the tube Sections were sta ned with hematoxyline-eosine Weigert s elast n or azan after microphotographs had been taken in the fluorescence m croscope to determine which type of vessels that was inn rvated

In attempts to increase the fluorescence of the adrenergic fib es in the tissues 3 cats were treated ith L-dopa (100 mg/kg s c) 2 hrs before excis on of the specimens Another 3 cats were given nialamid (Niamid® Pfi er 200 mg kg i p) 6 hrs before killing

Res rp ne (Serpasil® Ciba 5 mg/kg s c) was njected 24 hours before k lling to 3 cats to abolish the fluorescence of the adrenerg c fibres

To get an objective an lysis of the results blood essels of all sizes in the sect ons were photographed under the m croscope Measurements of essel diameters and counts of terminal bundles were then made f om the microphotographs

Results

Since the histochemical and pharmacological criteria for the specificity of the fluorescence reaction (Falck 1962 Corrodi and Hillarp 1963 1964 Dahlstrom and Fuxe 1964 a) are satisfied and the fluorescent fibres observed have the typical appearance of autonomic nerve terminals in general (*cf* Hillarp 1946 1959) and specifically of adrenergic terminal (*cf* Norberg and Hamberger 1964) there is no doubt that the fibres really represent adrenergic nerves (see Discussion) They will therefore for the sake of simplicity be referred to below as adrenergic fibres



Fig 3 *M gastrocnemius* of cat Longitudinal section A network of adrenergic fibres is present around small arteries (A) The accompanying vein (V) receives no adrenergic fibres Fluorescence microphotograph Magnification 1[illegible]

The fairly rich supply of adrenergic fibres to the intramuscular arteries and arterioles dominates the fluorescence microscopical picture in skeletal muscle (Fig 1 ?) the veins having no or only a scanty supply (Fig 1 2 9 No certain adrenergic innervation of the muscle fibres has been observed It must be pointed out however that the innervation of the vessels on the arterial side is by no means rich compared to that of such vessels in many other tissues (Dahlström and Fuxe 1964 b and unpublished observations in this laboratory

The adrenergic plexuses surrounding the vessels were directly superimposed on the smooth muscle layer (Fig 1 C) The terminals penetrated this layer only seldom



Fig 4A and B M tib alis anterior of cat Longitudinal secti n Adr arterioles In the lower figure single varicose fibres can be seen In of the arteriole (→) Fluorescence microphotograph Magnifi ati

even in the largest muscle arteries When penetration was obs present only in the outer part of the muscle layer The plex loose network of anastomosing strands in which there ran fine varicose terminals The adrenergic innervation thus h an autonomic ground plexus (Hillarp 1946 1959) The smal to a high degree oriented along the vessels (Fig 2 3 4) In were seen as intensely fluorescent dots (Fig 1 6 9) wh large in diameter probably due to superposition of fluor section (Fig 8) The individual terminals are difficult to de graphs due to their fineness and closeness (Fig 4B)

Arteries

The arteries entering the muscles showed a very sparse These vessels had elastic tissue showing a greenish aut disturbed the fluorescence microscopical picture This remained in animals treated with reserpine Nor did tr sodium borohydride (Corrodi Hillarp and Jonsson 19 cence in the vessels in contrast to the specific fluorescence after entering the muscles the arteries often came into mixed nerves containing both non fluorescent (sensory and



Fig 5



Fig 6

Fig 5 *M gastrocnemius* of cat Cross-section Several bundles of adrenergic terminals can be seen in relation to an arteriole (a) The accompanying venule (v) receives a single bundle of fibres (→) The internal elastic membrane of the arteriole shows nonspecific autofluorescence Fluorescence microphotograph Magnification 265 ×

Fig 6 *M tibialis anterior* of cat Cross-section Several bundles of adrenergic terminals are present around the external muscle layer of an artery The internal elastic membrane shows non-specific autofluorescence Fluorescence microphotograph Magnification 265 ×

axons with a weak fluorescence that increased in intensity as the nerve passed deeper into the muscle In view of the fact that the supply of adrenergic fibres to the arteries increased quite suddenly when the vessels passed into the muscle it seems reasonable to assume that a large part of the adrenergic innervation in the arteries derives from these fibres in the mixed nerves (*cf* Mitchell 1956) The adrenergic plexus around the larger arteries contained about 5 to 10 bundles of fluorescent terminals passing along and surrounding the vessel (Fig 1)

As the vessel diameter decreased from 200 μ down to about 10 μ at the precapillary level the number of bundles of adrenergic terminals successively decreased The arterioles did not possess a significantly higher degree of innervation than the arteries of larger diameter The small precapillary vessels (metarterioles) usually had only 2 diametrically localized bundles of terminals (Fig 8) The distance however between the bundles measured in cross-sections of the vessels was fairly constant about 15 μ for all the different sizes of vessel The general pattern of innervation was the same in small and large arterial vessels The innervation was uniform no definite accumulation of adrenergic fibres suggestive of a specialized sphincter innervation being observed in any section of the vascular bed not even at the bifurcations (Fig 2)

Veins

While the arterial vessels in the muscles exhibited a relatively abundant supply of adrenergic fibres the vessels on the venous side showed a very sparse innervation This holds true also for the veins going to the muscle Generally no adrenergic fibres at all could be seen around the intramuscular veins with inner diameters down to about 50 μ Typical intramuscular veins totally devoid of fibres can be seen in Fig 1 3 and 9 The accompanying arteries receive several bundles of adrenergic terminals In single larger intramuscular veins 100 μ a few fluorescent bundles of terminals could be seen at the circumference running along the vein These however were exceptions Adrenergic bundles were very seldom observed in middle-sized intramuscular veins with diameters of around 50 μ At the level of about 30 40 μ however a sparse but significant innervation appeared in some of the venules One or two bundles of terminals

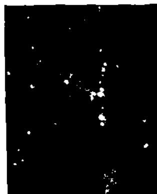


Fig 7



Fig 8

Fig 7 *M tibialis anterior* of cat Longitudinal section Two bundles of adrenergic fibres are present around a very small vessel (presumably a metarteriole) Fluorescence microphotograph Magnification 265 ×

Fig 8 *M gastrocnemius* of cat Cross-section Small blood vessels (presumably metaterioles) each with two bundles of adrenergic terminals Fluorescence microphotograph Magnification 265 ×



Fig 9 *M tibialis anterior* of cat Oblique section Several bundles of adrenergic fibres are present around the external muscle layer of the artery (A) The arteriole (a) also has a fairly rich supply of fibres The vein (V) receives no adrenergic terminals The internal elastic membrane of the artery shows non-specific autofluorescence Fluorescence microphotograph Magnification 165

could often be seen here passing along the vessel but the bundles of terminals were never as abundant as in the accompanying arterioles A typical venule with a single bundle of terminals can be seen in Fig 5 Owing to the difficulty of differentiating between arterioles and venules when transected longitudinally all quantitative estimations of the innervation was made from cross-sections The internal elastic membrane then made the arterioles easy to identify At the precapillary level however the amount of elastic tissue decreased so that it was often difficult to differentiate between precapillary and postcapillary vessels At this very small vessel size there were often seen two bundles of terminals of very intense fluorescence (Fig 7 8 It cannot be excluded

that some of these vessels lie on the postcapillary side. The capillaries presumably do not receive any adrenergic innervation as terminals were very seldom seen between the striated muscle bundles without any relation to larger blood vessels.

No significant differences in blood vessel innervation were observed in animals treated with L-dopa or nialamid as compared with untreated animals.

Discussion

All the histochemical criteria as formulated by Falck *et al.* (1962), Carlsson, Falck and Hillarp (1962), Falck (1962), Corrodi and Hillarp (1963, 1964) and Dahlström and Fuxe (1964 a) are fulfilled for the present method to visualize catecholamines and certain tryptamines in tissue sections. The green fluorescent fibres around the blood vessels in muscle disappear completely after postganglionic sympathetic denervation or reserpine treatment. Moreover, as the dopamine content of normal skeletal muscle is very low there is no doubt that the fluorescent fibres represent sympathetic noradrenaline-containing nerves (*cf.* Fuxe and Sedvall 1964).

The noradrenaline content in skeletal muscle can be reduced to about 10 [illegible] before the nerve fibres lose their fluorescence, which indicates a high sensitivity of the method (Fuxe and Sedvall 1964). In the present study, treatment of the animals with L-dopa and a monoaminoxidase inhibitor did not increase the number of visible fibres. Moreover, since there is a good correlation between the noradrenaline content in different organs and their supply of adrenergic fibres as observed with the present technique (unpublished data in this laboratory), we are justified in concluding that the method demonstrates the great majority of adrenergic fibres in a tissue section. No data have so far been obtained which speak against the view that the entire adrenergic innervation apparatus of blood vessels can be visualized.

A question that immediately arises is to what extent the adrenergic fibres around the blood vessels as found here represent vasoconstrictor nerves. There is a possibility that fibres to other effector organs accompany the blood vessels through the tissue. This, however, seems unlikely, as no other adrenergic effector system of quantitative importance has been found in skeletal muscle (Fuxe and Sedvall 1964). The whole composition of the adrenergic innervation with the network of fibres directly superimposed on the surface of the muscle layer clearly shows that most of the fibres represent vasoconstrictors (see for example Fig. 3). However, the possibility that a small proportion of the bundles of terminals passing along the arteries innervate structures other than blood vessels cannot be excluded.

The fine adrenergic nerve terminals with abundant varicosities (Fig. 4B) run in the anastomosing strands of a typical autonomic ground plexus, which in all probability represents the actual innervation apparatus (*cf.* Hillarp 1946, 19[illegible]; Falck 19[illegible]; Norberg and Hamberger 196[illegible]). In smooth muscle tissue this plexus usually forms a more or less dense network which penetrates everywhere between the cells so that the transmitter after its release can readily reach most — if not all — of the cells. In contrast to this, the adrenergic plexus innervating the intramuscular arteries, like all the arteries and veins so far studied in other tissues, does not penetrate in between the smooth muscle cells but surrounds and is directly superimposed on the outer surface of the muscle layer, even if this layer is thick. This finding agrees with electronmicroscopical studies of the innervation of blood vessels showing that no nerve fibres exist within that layer (Pease and Molinari 19[illegible]; Lever and Esterhuizen 19[illegible]; Rhodin 19[illegible]; Samara

singhe 1967 Appenzeller 1964) Thus not all cells in the muscle layer are reached by adrenergic nerve terminals This infers that the vasoconstrictor impulses cannot possibly be transmitted directly to all cells in the muscle layer

The electrical activity of single smooth muscle cells in the aorta and vena cava from turtles has recently been recorded with microelectrodes (Roddie 1962) This study indicates the occurrence of a direct intermuscular spread of excitation between the cells in the muscle layer — the transmission not being mediated by nerves It has been suggested from studies on pig carotid artery and renal vein (Burnstock and Prosser 1960 Prosser Burnstock and Kahn 1960) that electrical intermuscular transmission probably does not take place in mammalian blood vessels The large amount of connective tissue interposed between the smooth muscle cells in these vessels would present a high resistance to the flow of current However the possibility must still be considered that intercellular conduction takes place in small blood vessels where the amount of connective tissue is less abundant The chemically excited muscle cells in the vessels being in contact with the adrenergic fibres might thus activate the underlying cells by the electrical spread of excitation

Another possibility is that the adrenergic transmitter diffuses into the extracellular space between the muscle cells to activate them (*cf* Rosenblueth 1950) This general hypothesis for transmission in autonomic nerve-effector systems has been severely criticized by Hillarp (1946 1959) who obtained evidence against the view that transmitter diffusion over large distances is of significance for the transmission The blood vessels however may be exceptions owing to the special construction of their adrenergic innervation It has repeatedly been demonstrated that diffusion of the transmitter from vasoconstrictor nerves can take place in such a way that part of the released transmitter reaches the blood stream (Euler Luft and Sundin 1955 Rosell Kopin and Axelrod 1963 Vendsalu 1960 Haggendal 1963) It is probable that most of this transmitter leakage occurs from the smallest arteries and arterioles which have a muscle wall made up of only one or two layers of muscle cells and which answer for a major proportion of the blood vessels in muscle with a fairly rich adrenergic innervation If the transmitter leakage to the blood observed on vasoconstrictor nerve activation can be explained on this basis it is obviously impossible to draw any certain conclusions from this leakage as regards the physiological significance of transmitter diffusion for the transmission of vasoconstrictor impulses to the cells in the inner part of the muscle layer of larger blood vessels These cells might not be activated at all but function as an elastic core for the vessel The leakage observed raises however the possibility that noradrenaline relased from the arterial side of the circulation where the supply of adrenergic fibres is most abundant reaches the poorly innervated venous side of the circulation to act on the veins (see below)

The blood vessels entering and leaving the muscles exhibited a very poor adrenergic innervation — thus in contrast to several larger vessels as those to the skin and abdominal viscera the latter vessels having an abundant innervation on both the venous and the arterial side (Falck 1962 Dahlstrom Fuxe and Sedvall 1964) The regulation of blood flow to muscle thus presumably takes place almost exclusively inside this tissue

The very sparse adrenergic innervation of the venous side of the vascular bed in skeletal muscle was unexpected in view of the results of Mellander (1960 indicating a powerful vasoconstrictor innervation to the capacitance vessels in muscle In our study the intramuscular veins with diameters above 50 μ were almost totally devoid of adrenergic innervation Below this level however a sparse amount of adrenergic fibres

appeared in relation to the veins. The innervation was similar to that in the arteries with the fibres lying close outside the muscular coat. Only one or sometimes two bundles of terminals were found, however, running along the vessel and only about half the number of venules showed innervation. How then can we explain that activation of the very sparse innervation of the venules so profoundly and rapidly affects the capacitance function in muscle as found by Mellander (1960)? Several factors could be operating here. In veins where the wall lumen ratio is low even a slight shortening of the smooth muscles leads to a relatively great decrease in their volume (Mellander 1960). Moreover, since the venous pressure is low, even a few muscle cells and adrenergic terminals may well be enough to cause a considerable constriction of the venous vessels. The poor venous innervation might thus be sufficient to explain the effects observed on capacitance function. However, the possibility should be considered that part of the noradrenaline released from the arterial side of the circulation is transported by the blood stream to act on the venous side (see above). Due to the close juxtaposition of arterial and venous vessels also at the small caliber level, noradrenaline might also diffuse to the venules in the extracellular space. The venoconstriction produced by the direct venous innervation could thus be potentiated by noradrenaline deriving from the arterial innervation.

No direct observations have been made of how the adrenergic fibres reach the vessels but the close juxtaposition within the muscle of blood vessels and mixed nerves containing adrenergic fibres makes it possible that a large proportion of the fibres leave the mixed nerve to pass to the blood vessels at various levels after the entry of the muscle. The adrenergic fibres to the venules presumably do not pass down from the bigger veins, as no adrenergic fibres were generally observed around these vessels. The fibres could pass directly from the mixed nerves to the venules, or they might pass over from the arterial side to the veins via the capillaries. The capillaries presumably receive no adrenergic fibres as only very few were seen between the striated muscle bundles without any relation to larger vessels. It is possible that these single fibres pass over along capillaries to innervate venules.

In the present study the innervation seemed quite uniform over the whole arterial tree. No structures suggestive of selective sphincter innervations were to be found. Our findings do not, however, contradict the physiological evidence (Bucherl and Schwab 1952, Renkin and Rosell 1962) for a selective central control of the different sections of the vascular bed in skeletal muscle. As mentioned above, the adrenergic nerves may reach the vessels at various levels. Such an arrangement may provide the anatomical background for a functional differentiation in the vasoconstrictor control of the various sections of the vascular bed.

The present study has directly demonstrated the distribution of adrenergic nerves to arterial vessels and venules in skeletal muscle. The investigation has also pointed to the greatly varying organization of the adrenergic innervation to different vascular regions in the body. These variations make it extremely hazardous to draw any general conclusions as to vasoconstrictor innervation from studies on a single vascular region.

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Acta physiol scand 1965 64 87—92

From the Institute of Medical Physiology University of Copenhagen Denmark

The Magnitude of the Skimming Phenomenon in the Interlobular Arteries of the Cat Kidney

By

OLE I NISSEN

Received 9 September 1964

Abstract

Nissen O I *The magnitude of the skimming phenomenon in the interlobular arteries of the cat kidney* Acta physiol scand 1965 *64* 87—92 — Samples simultaneously collected from the arterial system from the deep renal veins and from the subcapsular renal veins in the cat kidney have been analysed with respect to red cell volume plasma proteins plasma osmolality (and plasma PAH) The ratio subcapsular venous concentration/arterial concentration was on an average 1 10 for the red cells 1 09 for the plasma proteins and 0 99 for plasma osmolality The ratio deep venous concentration/arterial concentration was on an average 0 90 for the red cells 0 89 for the proteins and 1 03 for the osmolality The difference between the protein ratios and a part of the difference between the hematocrit ratios may be attributed to the fact that a part of the fluid lost by ultrafiltration from the blood traversing the outer cortex is reabsorbed in the loops of Henle and the collecting ducts and thus will be added to the blood leaving the kidney by way of the deep veins A smaller part of the difference between the hematocrit ratios may be explained from the alterations in the tonicity of the blood during its passage through the kidney

In 1956 Pappenheimer and Kinter proposed their wellknown cell separation theory based on the phenomenon of plasma skimming described by Krogh (1929 p 6) This skimming should occur in the interlobular arteries the outer glomeruli then receiving a cell rich component of the blood and the deeper glomeruli a cell poor component.

A direct consequence of this cell-separation theory is that the blood leaving the superficial cortex should be cell rich and that the blood flowing from the inner part of the cortex (and the medulla) cell poor

According to v Möllendorff (1930 pp 133—135) the venous drainage of the cat kidney is characterized by the fact that the blood from a considerable outer part of the cortex passes to the well developed subcapsular veins which confluenting lead it along the surface of the kidney to the renal vein. The venous drainage from the medulla and the inner part of the cortex takes place as in other mammals through the arcuate veins

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veins with as many anastomoses of this sort as possible were always selected for puncture, and it is reasonable to assume that at the higher collection speed blood has been sampled from a more extensive area of the superficial cortex, but not from the renal vein.

The samples were generally collected during 3 to 5 periods per experiment. Three times 4 to 5 ml of blood was usually collected per period. Sometimes, when it was desired to make other analyses not reported here, the hematocrit determinations were omitted. Donor blood was administered slowly i. v., if the blood pressure tended to decrease during the sampling.

The catheters and syringes were repeatedly and slowly flushed with blood before starting the collection; however, when there was no interval between two periods the flushing was omitted.

A priming dose of PAH was followed by a sustaining dose administered continuously by an infusion pump. A minimum of half an hour elapsed before the first collection was initiated.

All the determinations were performed in duplicate. The plasma samples were analysed with respect to PAH by the method of Smith *et al.* (1945). The hematocrit was determined in Wintrobe tubes (1 000 g for 30 min) after careful mixing of the blood sample in the syringe. No correction for trapped plasma was made. The standard deviation determined from duplicates was 1.8 per cent of the mean (N = 36). As expected the hematocrit determination was a rather unexact method, and it is reasonable to suppose that a great part of the scattering of the points in Fig. 1 is due to the relatively large S. D. of this method.

The total protein concentration of the plasma was analysed by means of a biuret reaction (Gornall, Bardawill and David 1949). The standard deviation determined from duplicates was 0.6 per cent of the mean (N = 40).

The osmolarity of the plasma was determined by the method of freezing point depression (Advanced Instruments Inc.). The standard deviation determined from duplicates was 0.2 per cent of the mean (N = 33).

In most of the experiments urine was collected via a bladder catheter. The left ureter was ligated. The urine flow rates were purposely kept low: usually they amounted to about 0.1 ml per minute; consequently the loss of fluid by urine formation can have had only a negligible effect on the hematocrit and plasma protein concentration of the renal venous blood.

After the experiment the position of the deep venous catheter tip was determined. In all the experiments it was lying well (more than 0.5 cm) distal to the outlets of the subcapsular veins in the renal vein.

Results

The data presented derive from 11 expts. Protein concentrations as well as hematocrits were determined in 19 periods in 7 expts. (cf. p. 3 line 5—6). The results are shown in Fig. 1. The ratio between the protein concentrations of the deep or the superficial venous plasma and that of the arterial plasma (the "fractional concentration") is plotted as the abscissa, while the ordinate represents the corresponding ratio for the hematocrit values. The figure contains 2 more points for superficial than for deep blood, because valve formation at the catheter tip made collection of deep blood impossible in one of the 7 expts.

The average value for the fractional protein concentration in plasma from the subcapsular venous system was 1.09 and the average fractional hematocrit value in this blood was 1.10. The corresponding figures for the deep venous blood were 0.89 and 0.90.

The long dashed curve represents the relationship between the ratios to be expected if the only factor causing deviations of the ratios from one were a net removal of a protein and erythrocyte free isotonic fluid from the arterial blood or a net gain of such fluid. The average hematocrit value 34 per cent (range 23—44) was used for calculation of the curve. The slope of the curve is less than 45° because such fluid loss or gain would affect only the plasma volume and because the hematocrit is a concentration per unit volume of total blood whereas plasma protein concentration is given per unit volume of plasma; for the same reasons the curve is curvilinear.

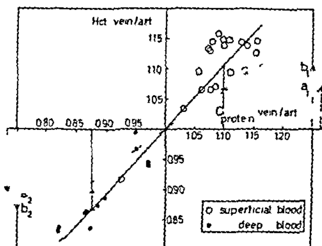


Fig 1 Abscissa o concentration of protein in subcapsular venous plasma/concentration of protein in arterial plasma ● concentration of protein in deep venous plasma/concentration of protein in arterial plasma

Ordinate o hematocrit in subcapsular venous blood/hematocrit in arterial blood ● hematocrit in deep venous blood/hematocrit in arterial blood

For details confer the text

The fractional osmolality of the plasma samples (venous/arterial) was determined in 7 periods In the superficial plasma this figure was on an average 0 99 (range 0 96—1 00) in the deep plasma 1 03 (range 1 00—1 06) The difference between the two types of venous plasma may in all essentials be accounted for by differences in the concentrations of sodium ions with matching anions (unpublished data) As the erythrocyte membrane is virtually impermeable for sodium ions a hypotonicity in the plasma due to a lowering of the concentrations of these ions will cause a swelling of the erythrocytes

Glomerular filtration as such will cause a movement of the hematocrit protein concentration relationship from point 1 0—1 0 in Fig 1 along the dashed curve towards the right Subsequent regain of an isotonic fluid will lead to a backward displacement along the same curve towards or past the 1 0—1 0 point

However the fact that the venous blood is non isotonic indicates that the fluid which the blood — upon filtration — has regained by reabsorption has been non isotonic viz hypotonic in the case of the superficial blood and hypertonic in the case of the deep blood

Consequently in the former case the covariation of the hematocrit ratio and the protein concentration ratio will be described by a curve with a smaller slope than the dashed curve since a swelling of the red cells takes place A correction for this fact — based upon the average fractional osmolality — has been applied in Fig 1 (upper dotted curve) Similarly a correction for the hypertonicity of the deep blood has been applied (lower dotted curve)

The full-drawn line represents the relationship between the ratios estimated from the experimental data The ratio $a_1 + a_2/b_1 + b_2$ gives a rough measure for that part of the difference between the hematocrit ratios which can be accounted for by processes other than skimming

In 11 periods in 7 expts the arterial plasma concentrations of PAH were below 5 mg per cent The average extraction fraction (defined as 1 — concentration of PAH in venous plasma/concentration of PAH in arterial plasma) for PAH in the superficial venous plasma at these concentrations was $0\ 94 \pm 0\ 02$ (S D n = 11) the corresponding value for the deep venous plasma was $0\ 82 \pm 0\ 04$ (S D n = 11) In one additional experiment the plasma concentration was below 5 mg per cent (namely 2 1 mg per cent) but in this the extraction fractions (0 73 for the superficial plasma and 0 55 for the deep plasma) deviated so much from the others that these values were omitted in the calculations of the averages and standard deviations

The average blood pressure was 125 mm Hg (range 92—152)

Discussion

From the difference between the concentrations of plasma proteins plasma PAH and red corpuscles in the superficial and deep venous blood it is concluded that the areas drained by the two venous systems differ considerably with respect to function

The very high extraction fraction of PAH (94 per cent) in the plasma drained from the peripheral part of the cortex indicates that at most 6 per cent of this plasma may pass through nonextracting tissues or Pappenheimer shunts Further discussion of the PAH extraction is postponed to a subsequent paper

According to the cell separation theory of Pappenheimer and Kinter (1956) the hematocrit of the blood leaving the peripheral parts of the cortex should be considerably higher than that of arterial blood A single sample of blood from a subcapsular vein of the cat kidney was examined by these authors (p 383 in their paper) they reported the hematocrit to be normal but did not discuss the implications for the theory

Ulfendahl (1962) found the hematocrit values of subcapsular venous blood to be some 10 per cent higher than those of arterial blood He considered this as due to plasma skimming however he pointed out that the percentage difference between the hematocrit values was much too small to fit into the cell separation theory of Pappenheimer and Kinter Samples form the deep renal venous system were not investigated

The present experiments confirm that the hematocrit value of the blood drained from the peripheral cortex is higher than that of arterial blood and add complementary information on the hematocrit of deep venous blood

However a different conclusion has been arrived at from the present experiments because factors other than skimming that may affect the venous hematocrit values has been taken into consideration

These are the following

1) The blood plasma traversing the outer part of the cortex may loose more fluid by glomerular ultrafiltration than it gains by reabsorption and the opposite may hold for the blood plasma passing through the deep parts of the kidney This would *alter the hematocrit and the plasma protein concentration in the same direction* and the alterations would be correlated as indicated by the long dashed curve in Fig 1

2) If the blood traversing the outer or the inner part of the kidney regains — by reabsorption — a fluid which is hypotonic or hypertonic (relative to arterial blood) a movement of water between the red cells and the plasma would take place The

occurrence of this phenomenon in the kidney is indicated by the fact that the osmolalities of the deep and superficial venous blood differ significantly from that of arterial blood By further taking this factor into consideration (using the average osmolalities of superficial and deep venous blood) the fractional hematocrits and the fractional protein concentrations should be correlated as indicated by the two short dotted curves in Fig 1 — provided no skimming occurred

(The author has disregarded the formation of renal lymph as a factor causing any significant changes in the hematocrit and protein concentration of the renal venous blood because of the smallness of the lymph flow in comparison with the renal blood flow)

By comparing the distances $a_1 - a_2$ and $b_1 - b_2$ in Fig 1 it may be seen that the two above mentioned phenomena account for the major part of the observed rather small deviations of the fractional hematocrits from one From this it must be concluded that plasma skimming in the interlobular arteries as proposed by Pappenheimer and Kinter takes place at most to an negligible extent

The auth r is greatly indepted to professor Poul Kruhı ffer for criticism of the manuscript.

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Acta physiol scand 1965 64 93—105

From the Department of Physiology Karolinska Institutet Stockholm Sweden

The Effect of Osmotic Pressure Changes on the Isolated Muscle Spindle

By

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Abstract

Ottoson D *The effect of osmotic pressure changes on the isolated muscle spindle* Acta physiol scand 1965 *64* 93—105 — The action of hypertonic and hypotonic solutions on the sensory endings of the muscle spindle has been studied in isolated preparations It was found that changes of osmotic pressure by more than 25 per cent caused an augmentation of the activity of the resting spindle and modified its sensitivity to stretch Increase of pressure produced a discharge the peak frequency of which increased linearly with osmotic strength The maximum effect was usually obtained with concentrations of four to five times that of Ringer s solution The responsiveness of the spindle to stretch decreased with increasing pressure Decreasing the osmotic pressure produced a short lasting increase of the activity of the resting spindle followed by a decline and final cessation of the discharge The responsiveness to stretch was increased during the period of repetitive firing and then decreased Washing with Ringer s solution after immersion in hypotonic solutions regularly produced a prolonged discharge and a reduced sensitivity to stretch It is concluded that the observed changes are mainly due to osmotic effects on the sensory endings of the spindle The results suggest that the endings behave as simple osmotic sacs within a limited range of osmotic strengths and that the sensory membrane is equally sensitive to distention and folding

The muscle spindle responds with a burst of impulses when stretched or when the intrafusal fibres contract It is generally accepted that this response arises as a result of the deformation of the endings of the afferent fibre At present but little is known about the kinetics of the mechanical events underlying the excitatory process There seems to be reason to believe that the conversion of the mechanical stimulus into the graded electrical response (Katz 1950 a b) occurs in the membrane of the bulbous expansions of the ramifications of the nerve fibre (Katz 1961) Since the end bulbs vary in size as well as in their structural relation to the intrafusal fibres it seems most unlikely that all endings are affected to the same extent by the elongation or shortening of the muscle fibres The sustained potential recorded from the nerve during stretch can therefore be presumed to represent the total integrated effect of a differential distortion of various

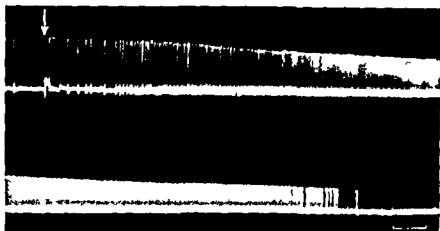


Fig 1 Typical effect of strong hypertonic solution Continuous record on moving film A arrow one small drop of a 5 × R sucrose solution was added to the bath Time calibration 1 sec

parts of the sensory membrane In view of the structural organization of the spindle it may also be assumed that the external stimulus undergoes a transformation before the sensory endings are reached The parameters of the external stimulus consequently represent an inaccurate measure of the characteristics of the actual stimulus

It was thought that by changing the osmotic pressure of the external solution it would perhaps be possible to obtain a uniform and also a quantitatively more well defined stimulus than by stretch As will be reported in the present paper it was found that the spindle could be excited by osmotic pressure changes and also that characteristic alterations of the sensitivity to stretch were produced by anisotonic solutions A preliminary report of the results has been published earlier (Ottoson 1964)

Methods

Preparation The experiments to be described were carried out on isolated spindles of the frog s *m ext di long II* Dissection was made under dark field illumination with the muscle mounted at resting length in a Ringer bath (Ottoson 1961 1964) After being localized by careful teasing of the muscle fibres the spindle was cleared from adjacent tissues The bundle of intrafusal fibres was cut at some distance from the ends of the spindle and the preparation lifted over into a small chamber (0 5 2 mm) where it was clamped at each end to a thin plastic rod One of the rods was connected to a micromanipulator so that the length of the muscle could be changed

In order to prevent contractions or changes in the tonus of the intrafusal fibres the muscle bundle was crushed close to the ends of the spindle Sometimes the fibres also had to be punctured by a glass capillary so as to eliminate fibrillations Stretch of 250 msec or 3 sec duration was applied by means of an electromagnet connected to the spindle by one of the plastic rods Before exposure to the test solution the response to stretch was recorded at regular intervals and no test were made until the response in Ringer s solution had remained constant for at least 10 min

Recordings The impulses of the afferent nerve were recorded with silver chloride agar electrodes One electrode was placed in the fluid in the chamber while the other was applied to the sensory axon which was lifted up in oil The electrodes were connected to a Grass P 6 amplifier and a Tektronix 502 oscilloscope DC recording was used in all experiments with test stretches

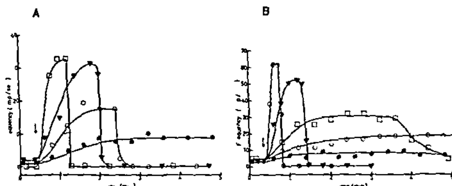


Fig 24 Effects of hypertonic sucrose solutions of different concentrations. O dinate average frequency of discharge Change from iso- to hypertonic fluid marked by arrow Concentrations expressed relative to Ringer's fluid ●—● = 1 5 × R O—O = 2 × R ▼—▼ = 3 × R □—□ = 5 × R
B Effect of hypertonic NaCl Ringer's solution Concentrations ●—● = 1.25 × R O—O = 1 5 × R □—□ = 2 × R ▼—▼ = 3 × R ◑—◑ = 6 × R

Solutions The normal Ringer's solution was the same as that used by Adrian (1956) Hypertonic solutions were made by adding sucrose or salts to Ringer's solution. When sucrose was used a 270 mM solution was assumed to be osmotically equivalent to Ringer's fluid Buffering was produced by 2 mM sodium phosphate buffer at pH 7 2 Sodium free solutions were made up by replacing sodium with choline chloride on a mole for mole basis All solutions were made up fresh before use Hypotonic solutions were made by adding distilled water to Ringer's solution the calcium concentration being kept at normal value by adding $CaCl_2$ to the diluted solution Concentrations of the solutions used are given relative to Ringer's solution (cf Howarth 1958) thus a hypertonic solution of twice the normal osmotic pressure is denoted as 2 × R a hypotonic solution of half the normal pressure as 0 5 × R. When the effect of a solution was to be tested part of the fluid in the chamber was first removed and then replaced by the test solution To obtain a complete replacement of the Ringer's fluid several changes had to be made This could usually not be carried out in less than half a minute It was therefore not possible to determine the accurate time course of the initial action of the test solutions After being soaked in the test solution the spindle was left to recover in Ringer's fluid for 20 to 30 min before the next run In some experiments the effect of adding a small drop of high concentration to the bath was also studied With this procedure the gradually developing effects of the added solution could be followed (see e g Fig 1) the final concentration however remaining unknown All experiments were carried out at room temperature

Results

Hypertonic solutions

Effects on the resting spindle The change from Ringer's solution to a hypertonic solution regularly elicited an increased activity of the spindle (Fig 1) This effect in the following called the hypertonic discharge was usually not obtained until the osmotic pressure was raised to 1 5 × R In some preparations however an increase to 1 25 × R was sufficient to increase the firing rate of the spindle The effect appeared within 10 to 30 sec after the change to the hypertonic solution With 1 5 R the discharge increased slowly to 10 to 15/sec and in most cases remained constant at this frequency for several minutes With further increase of concentration the response appeared with shorter latency the rise became faster and the peak frequency higher (Fig 2A) Typically the

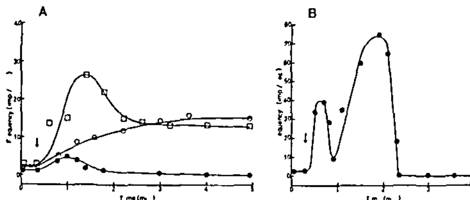


Fig 3 *A* Effect of Na concentrations on the hypertonic discharge Responses obtained in one preparation treated with sucrose Ringer's solution (○—○) NaCl Ringer's solution (□—□) and sodium free sucrose solution (●—●) made up by adding sucrose to Ringer's solution the NaCl of which was replaced by choline chloride The solutions were of equal osmolarity (2 × R) and applied in the order ○—○ □—□ ●—● A final control test with sucrose Ringer's solution gave a response identical to that shown by the curve ○—○
B Double discharge evoked by a hypertonic (6 × R) NaCl Ringer's solution

duration of the discharge also became successively shorter as the osmotic pressure was increased With concentrations above 2 × R the firing generally stopped abruptly and the spindle then remained silent as long as it was immersed in the hypertonic solution The maximum response in terms of peak frequency was most often obtained with 3 to 4 R but sometimes not until the concentration was raised to 6 × R The increased activity of the spindle was usually associated with an increased frequency of miniature potentials (Katz 1950 a)

In order to ascertain that the observed effects were not due to contractions of the intrafusal bundle the preparation was observed under the microscope in a number of experiments during and after the change from isotonic to hypertonic solution in the bath In no case was it possible to detect any contractions or movements of the fibres which could explain the excitatory effect The fact that in all experiments the muscle fibres were pinched close to the polar ends of the spindle also reduces the likelihood of muscular contractions as the cause of the afferent discharge

Hypertonic NaCl solutions had essentially the same effect as the sucrose solutions (Fig 2B) In general however the NaCl solutions were active in lower concentrations than the sucrose solutions With NaCl solutions the effect also appeared earlier and the frequency of the discharge rose faster than with sucrose solutions of corresponding osmolarity It was also possible to drive the spindle to higher frequencies with NaCl solutions than with sucrose solutions When the osmotic pressure was raised by adding choline chloride to Ringer's fluid the hypertonic effect was similar to that obtained with sucrose

The influence of the Na concentrations of the bathing fluid on the hypertonic effect was examined in a series of experiments in which the spindle was exposed to hypertonic sucrose solutions with different Na concentrations the NaCl being replaced by choline chloride It was found that a lowering of the Na concentration be

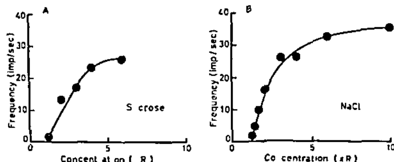


Fig 4 Relat on between osmotic pressure and discharge Ordinate peak frequency of discharge Abscissa osmotic pressure in units relative to Ringer s solution Results obtained from two different preparations *A* sucrose Ringer s solution *B* NaCl Ringer s solution

low the value of normal Ringer s solution was regularly followed by a diminution and shortening of the hypertonic effect (Fig 3A)

With high concentrations the spindle sometimes responded with two bursts instead of a single discharge (Fig 3B) The initial discharge was usually brief and separated from the second more prolonged one by a short interval of lower firing rate In some preparations there was a silent pause in between the two bursts The double response was most often seen with hypertonic NaCl solutions but also appeared with sucrose solutions

Recovery After treatment with low concentrations the spindle recovered completely when returned to Ringer and repeated exposures were usually followed by only a small decline of the response provided the spindle was left to recover in Ringer s solution for sufficient time after each treatment After exposures to high concentrations (above 2 × R) recovery was less complete and the response to subsequent applications became successively smaller Repeated treatments with solutions above 2 × R were also typically accompanied by a gradual reduction of the duration of the response

To minimize exhaustion of the spindle the solutions to be tested were applied in the order from lower to higher concentrations When strong solutions were used the time of exposure was kept as short as possible After each exposure the spindle was left for at least 20 min in Ringer s fluid and with higher concentrations for 30 to 40 min between the exposures By these precautions the deleterious effects of repeated immersions could be minimized

Relation between osmotic pressure and discharge Since the spindle did not recover completely after immersion in strong solutions it was difficult to establish the relation between the osmotic pressure and the increase of activity of the spindle This difficulty could be partly overcome by prolonging the recovery period in Ringer s fluid and shortening the soaking time Some preparations were also more resistent than others and in these cases it was possible to obtain comparable data for a relatively wide range of concentrations The curves in Fig 4 give examples of the results obtained in two such experiments As seen the peak frequency of the discharge rises almost linearly with increasing osmotic pressure up to 4 × R The curves also illustrate the typical differences between the effects of sucrose and NaCl

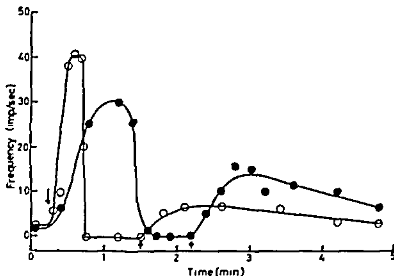


Fig 5 Post hypertonic effects on return to Ringer's fluid after soaking in NaCl Ringer's solution Change from iso- to hypertonic solution marked by first arrow from left return to Ringer's fluid by second and third arrow respectively Concentrations relative to Ringer's fluid ●—● = 3 × R ○—○ = 5 × R

Post hypertonic effects The behaviour of the spindle when soaked in Ringer's solution after exposure to increased osmotic pressure varied with the concentration of the hypertonic solution and with the time of exposure A spindle that had been treated with a weak hypertonic solution for a few minutes usually returned to its original spontaneous firing rate within 10 to 20 sec whereas after prolonged immersion the recovery took place more slowly In many preparations however washing with Ringer's solution produced an increased activity (Fig 5) which was sometimes maintained for several minutes This type of response which varied from one preparation to another was obtained only after treatment with strong hypertonic solutions and only when the change from the hypertonic solution to Ringer's fluid was made after cessation of the hypertonic discharge In the experiments illustrated in Fig 5 3 × R gave a stronger off response than 5 × R In other experiments the intensity of the discharge increased with increasing osmotic pressure

Effects on the response to brief stretch Increase of the osmotic pressure of the bathing solution regularly caused a reduction of the response of the spindle to stretching This effect was noticeable at a 50 per cent increase of the osmotic pressure and became gradually more pronounced with stronger solutions (Fig 6) In the initial phase of the action of strong solution when the spindle discharged vigorously stretch usually failed to produce any response (Fig 6 l) In the later phase of the hypertonic discharge when the activity had declined stretch regularly caused an inhibition of the maintained discharge Still later after cessation of the hypertonic discharge stretching usually elicited one single impulse at the onset of stretch (n)

The time course and magnitude of the change of the response in hypertonic media are illustrated by the curves in Fig 7 The responses are expressed as percentages of

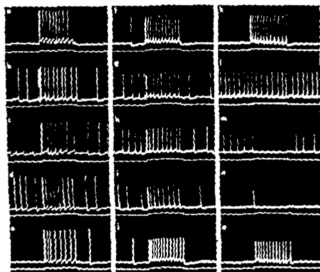


Fig 6 Typical effects of hypertonic solutions of response of muscle spindle to brief (250 msec) stretch *a* response in Ringer s fluid *b—d* after 0 5 2 and 5 min in NaCl Ringer s solution (1 5 × R) *e* 5 min after return to Ringer s fluid *f—j* similar trials with × R and *k—o* with 3 × R solutions Duration of stretch indicated by lower tracing

the number of spikes elicited in Ringer s fluid by the test stretch When calculating the effect of the various solution tested the average number of spikes of the hypertonic discharge in a period corresponding to that of stretch was subtracted from the total number of spikes in the stretch period In addition to the curve showing the change of the response the curve of the hypertonic discharge also is given in the figures The curves in A and B give two examples of the reduction of the response in a 1 5 × R solution With solutions 2 to 3 times the normal osmotic pressure the depressive effect became still more pronounced as illustrated by the curves in C and D As seen there is still a residue of 15 to 20 per cent of the original response in 2 × R while in 3 × R the block is complete It may also be noted that return to Ringer s fluid is followed by a transient phase of increased responsiveness

Hypotonic solutions

Effects on the resting spindle Immersion in solutions of low osmotic pressure gave rise to a transient increase of the activity of the spindle In most cases this effect was not obtained until the osmotic pressure had been reduced to 50 per cent of that in Ringer s fluid but sometimes a reduction of 25 per cent was sufficient to elicit a response As a rule the discharge frequencies of the hypotonic responses were lower than those of the hypertonic responses with a 0 5 R solution the discharge usually did not exceed 15 sec As the osmotic pressure was further reduced the response increased (Fig 8) with 0 1 × R solution firing rates of 20 to 40 sec were obtained With diminishing osmotic pressure the duration of the response also became successively shorter A 0 5 × R solution usually produced an increased activity for 1 to 1 5 min followed by a slow decay while a 0 1 R solution usually gave a short burst of impulses after which the spindle remained silent as long as it was kept in the solution The type response described above was obtained in 40 out of the 44 spindles tested with hypotonic solutions In the remaining 4 spindles application of hypotonic solutions produced a decline of the

Fig. 7. Effects of hypertonic NaCl Ringer's solutions on the response to stretching (open circles) and on the activity of the resting spindle (filled circles). A and B 1.5 × R. C 2 × R and D 3 × R. Hypertonic periods between arrows. Response values expressed in per cent of number of impulses elicited by test stretch in Ringer's fluid.

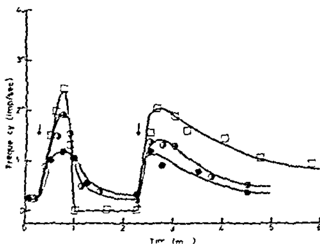


Fig. 8. Effects of hypotonic solutions. Ordinate: average frequency of discharge. Change from iso- to hypotonic solution marked by first arrow from left, return to Ringer's fluid by second arrow. Concentrations relative to Ringer's fluid: ●—● = 0.5 × R; ◑—◑ = 0.25 × R; □—□ = 0.1 × R.

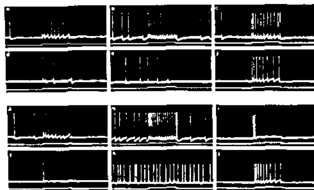


Fig 9 Typical effects of hypotonic solutions on response of muscle spindle to brief (250 msec) stretch *a* response in Ringer s fluid *b*—*d* after 0.5 2 and 5 min in 0 5 × R *e* and *f* 0 5 and 3 min after return to Ringer s fluid Records *g*—*l* similar run with 0 25 × R Duration of stretch indicated by lower tracing

activity Since these spindles maintained an unusually high spontaneous firing rate (8 to 12/sec) in Ringer s fluid it seems most likely that they had been damaged during the dissection procedure It is noteworthy that though these spindles did not respond to a reduction of the osmotic pressure they reacted quite normally to hypertonic solutions

Post hypotonic effects The change from hypotonic to isotonic fluid regularly elicited an increased activity of the spindle (Fig 8) The response appeared within a few seconds after return to Ringer s fluid and was maintained for several minutes With successively diminished pressure the discharge became more intense on return to Ringer s solution The firing rates were usually not as high as those obtained by application of hypotonic solutions The intensity as well as the duration of the discharge also varied with the time of exposure

Effects on the response to brief stretch The records in Fig 9 illustrate the typical effects evoked by soaking the spindle in hypotonic solutions Immersion in a 0 5 × R solution produced the usual increase of activity followed by a final cessation of the discharge As shown by record *b* the response to stretch is enhanced in the period of hypotonic discharge As the latter decays the response diminishes and after cessation of the hypotonic discharge stretch only produces a few impulses (*d*) Return to Ringer s fluid gave the usual effect i.e. a short lasting increase of activity Stretch applied in this period often failed to produce any noticeable effect on the spindle or elicited only a few impulses (*e*) With the decline of the posthypotonic discharge the response appeared again attaining its original size in about 5 min With further decrease of the osmotic pressure of the external fluid the effects on the spindle became more marked The enhancement of the response in the period of hypotonic disharge (*h*) was followed by a pronounced reduction until only one impulse was left (*j*) Washing in Ringer s fluid elicited an intense post hypotonic discharge which persisted unchanged during stretching (*k*) The time course and magnitude of the effects elicited with hypotonic solutions appear in detail from Fig 10

The curves in Fig 11 summarize the results obtained in the present study Curve A illustrating the excitatory action of solutions of different osmotic strengths gives the mean values from 16 experiments in which the recovery of the spindle after each of the exposures was almost complete thus allowing tests to be carried out within the range from 4 R to 0 25 R As appears from curve A [illegible] side of the

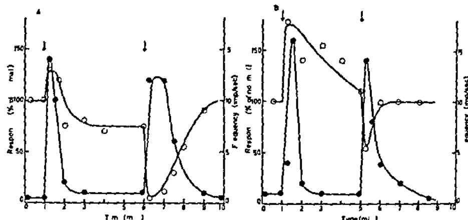


Fig 10 Changes of response to stretch (open circles) in 0 5 × R (A) and 0 25 × R (B) Filled circles show changes in frequency of maintained discharge Response values expressed as in Fig 9 Hypotonic periods between arrows

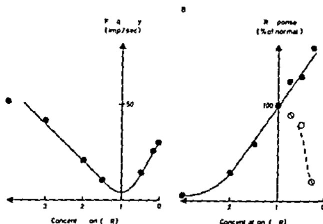


Fig 11 A relation between osmotic pressure and discharge of spindle Composite results of 16 experiments (8 with hypertonic and 8 with hypotonic solutions) Ordinate peak frequency of discharge Abcissa osmotic pressure of the bathing fluid B responsiveness of muscle spindle at different osmotic pressures Ordinate response expressed in per cent of that obtained in Ringer's fluid as in Fig 9 Abscissa osmotic pressure of bathing fluid in units relative to Ringer's fluid Dotted line shows late decrease of sensitivity in hypotonic solutions

isotonic point lead to an increased activity Though a comparison between the effects of increased and decreased pressure is limited to a small range the results suggest that osmotic changes of the same order of magnitude in opposite directions have about the same action This indicates that the sensory membrane is equally sensitive to folding and distention Curve B illustrates the responsiveness of the spindle to stretch when kept in solutions of different osmotic pressures As seen the response of

the spindle to a given stretch is linearly related to osmotic strength within the range from 2 × R to 0 25 × R The close correlation between excitatory action and responsiveness on one hand and osmotic pressure on the other indicates that the changes underlying the action of anisotonic media closely follow the laws governing osmotic processes

Discussion

The experiments described above demonstrate that changes in osmolarity have an excitant action on the muscle spindle when the pressure is raised or reduced by more than 25 per cent of that of Ringer s solution As also shown increased osmotic pressure results in a reduced sensitivity to stretch while reduced pressure gives rise to a transient increase followed by a decrease in responsiveness Likewise return back to Ringer s solution after immersion in anisotonic media excite the spindle and produce changes in its responsiveness to stretch The simplest explanation of these effects seems to be that they arise as a result of a mechanical deformation of the sensory endings caused by volume changes of the different tissues forming the spindle The effects may also be produced by contractions of the intrafusal fibres but this explanation is less likely *since the muscle fibres were always pinched before exposing the spindle* to the test solution Also direct observations of the spindle did not in any case disclose any contractions

As shown by Katz (1961) in an electronmicroscopical study of the frog s muscle spindle the terminal branches of the sensory fibre form bulbous expansions which are seated in cup-like depressions of the muscle fibres to which they are attached by thin strands of connective tissue while other bulbs are lying free in the lymphatic space Out of the number of factors which may contribute to the effects observed the volume changes of the sensory end bulbs and the intrafusal fibres are probably the most important Since many of the sensory bulbs are only loosely attached to the muscle membrane it does not appear to be very likely that the excitatory effects and the changes in sensitivity to stretching are primarily to be ascribed to the volume changes of the muscle fibres The conclusion would consequently be that the changes in activity of the spindle when exposed to anisotonic media are mainly due to osmotic effects on the sensory end bulbs

In addition to the direct osmotic effect on the sensory endings changes in osmotic pressure will also affect the afferent fibre It has been demonstrated (Stampfli 1955 Stampfli and Nishie 1955 Schmidt and Stampfli 1959) that hyperosmotic media produce a reduction of the membrane potential of myelinated fibres whereas hypo-osmotic media have the opposite effect It is of interest to note that the hypotonic effect was also obtained by switching from a hypertonic solution back to isotonic solutions (Schmidt *and Stampfli 1959) It has further been demonstrated by Sato (1950) that a nerve* treated with hypertonic media responds to a constant current with a repetitive discharge If it is assumed that the excitant action is caused by the distortion of the sensory membrane it seems most likely that this action is modified by the changes in the membrane potential of the nerve

The changes in sensitivity to stretch may be explained in a similar way When soaking the spindle in a hypertonic medium the sensory endings shrink as water leaves the cells and the membrane becomes folded Stretching a hypertonic spindle can be

assumed to result in the opposite effect *i e* a distention of the membrane In the case of a weak hypertonic action stretching would produce a response while in a strong hypertonic medium stretch would result in an unfolding of the membrane and consequently lead to a reduction of the stimulus as represented by the folding In this case stretch would be expected to cause an inhibition of the hypertonic discharge As described above this effect was observed during a transient phase of the hypertonic period when the spindle was bathed in strong hyperosmotic media The mechanisms underlying the changes in sensitivity to stretch in hypotonic media seem to be analogous to those responsible for the hypertonic effects However the events in hypotonic media are no doubt more complex since in this case the osmotic action is influenced by the effect caused by deficiency of ions essential to the function of the spindle The effect of hypotonic solutions is similar to that caused by stretch insofar as the sensory membrane is distended in both cases The situation when the hypotonic spindle is being stretched may thus be compared with what happens when a short stretch is superimposed upon a sustained stretch The increase of the response in the initial phase of the hypotonic period would accordingly correspond to the increased responsiveness of the spindle under maintained stretch

If the sensory endings behaved as ideal osmometers it would be possible to calculate the volume changes of the sensory endings It has been demonstrated (Sato 1954 Reuben *et al* 1963) that there is a linear relation between osmotic strength and relative volume of single muscle fibres in hypo-osmotic media Recent studies on the effect of hypertonic solutions on single fibres (Dydynska and Wilkie 1963) have further shown that a similar relationship exists up to 2 × R This correlation is absent in stronger solutions probably because of damage to the fibres The results obtained in the present study indicate that within a limited range of osmotic strengths the sensory endings behave as simple osmotic sacs In assessing the effects observed it has however to be taken into account that the changes in volume are not due to passage through the membrane of water alone Together with the flow of water there is most likely also an exchange of ions (Boyle and Conway 1941) between the outer media and the cell interior leading to changes in the potential gradient across the membrane It has been demonstrated (Stampfli and Nishie 1955 Schmidt and Stampfli 1959) that hypertonic media cause a decrease of the permeability of myelinated fibres to sodium and potassium and hypotonic media an increased potassium permeability Similar changes may also occur in the end bulbs and in the afferent fibre and interfere with the depolarization caused by the mechanical distortion There seems to be but little doubt however that the essential event underlying the action of anisotonic media on the spindle is the transfer of water through the sensory membrane Teorell (1959 a b 1962) has developed n electrokinetic model of a mechanoreceptor which seems to have interesting bearings on the phenomena observed in the spindle In this model excitation is suggested to occur as a result of a sequence of changes of the gradients of osmotic pressure ionic concentration and electrical potential across the membrane the most important change being the electro-osmotic flow of water through the membrane It is not yet possible to determine to what extent the proposed functions of this electrohydraulic analogue are applicable to the events taking place in the sensory end bulbs In the case of the muscle spindle several other factors may be involved and the final effect as represented by the discharge and the change in sensitivity to stretch can be assumed to be determined by a complex interplay of factors affecting a number of different functions and structures

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Acta physiol scand 1965 64 106—108

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The Effect of Small Doses of Chemically Pure Secretin on the Volume and Bicarbonate Output of the Pancreatic Juice in Dogs

By

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Received 10 September 1964

Abstract

Säteri H L Thulin and B Welander *The effect of small doses of chemically pure secretin on the volume and bicarbonate output of the pancreatic juice in dogs* Acta physiol scand 1965 64 106—108 The volume and bicarbonate output of the pancreatic juice during fasting and during infusion of chemically pure secretin at doses without effect on the liver were studied by a non-surgical method Eight experiments were made in 2 unanesthetized dogs The doses ranged from 0 25 to 1 5 clinical units per kg b w and hr At the smaller doses there was only a slight increase of the volume and the bicarbonate output At the larger doses the maximum volume 25 ml per 30 min was about 3 times the initial and the maximum bicarbonate output 1 4—2 7 meq per 30 min about 50 times the initial in one dog and about 10 times in the other

Recently chemically pure preparations of secretin have been made available for studies of its effect on the pancreas and the liver Doses below 1 5 clinical units per kg b w and hr of the chemically pure secretin Secretin Vitrum (Jorpes and Mutt 1961 1962) have no effect on the output of hepatic bile in dogs and the choleretic action of secretin in dog is thus much less than that of bile acids (Jonson and Thulin 1964)

The present study was undertaken in order to evaluate the effect on the pancreas in dogs of this chemically pure secretin at small doses without effect on the output of hepatic bile

Procedure

2 male mongrel dogs (dog A weight 16 kg and dog B weight 14 kg) were given 2 hr infusions of the chemically pure secretin Secretin Vitrum prepared by Jorpes and Mutt It contains *17 500 clinical units of secretin per mg substance*

The pancreatic juice was collected according to Säteri (1963) A tube consisting of 2 tubes of different lengths was inserted into the stomach of the dog The tube was passed through one of the nostrils and the upper part was fixed on the head of the dog The distal end of the longer tube was overnight carried by the peristalsis into the duodenum and the shorter tube remained in the stomach The position was checked with X ray immediately before the experiment The stomach was kept empty by continuous suction through the shorter tube The pancreatic juice was collected by suction with a syringe and the volyme was measured each half hour The bicarbonate content was determined by titration

TABLE I Mean pancreatic juice volume and b carbonate output during basal experiments (exp 1) and during initial periods of secretin experiments (exp 2—4)

	Experiment			
	1	2	3	4
Volume ml per 30 min				
Dog A	15 3	7 1	18 5	8 1
Dog B	14 6	9 3	9 8	8 0
B carbonate meq per 30 m n				
Dog A	0 245	0 138	0 191	0 055
Dog B	0 155	0 198	0 146	0 260

TABLE II Mean pancreatic ju ce volume and mean b carbonate output during administration of secretin

	Dose clin cal units per kg bw and hr					
	0 25	0 50	0 75	1 00	1 25	1 50
Volume multiples of initial						
Dog A	1 6	1 4	2 2	1 9	3 6	3 3
Dog B	1 4	1 4	2 2	3 0	2 6	3 3
B carbonate multiples of initial						
Dog A	1 7	5 1	16 9	18 0	7 6	49 0
Dog B	1 1	4 1	3 3	9 9	6 1	7 8

4 experiments 1 basal and 3 with secretin were made in each dog Each experiment lasted for 6 hrs In the *basal e perime t* (exp 1) no secretin wa given E ch *sec tin expe ment* was divided nto 3 periods of 2 hrs During the initial 2 hr per od only sal ne was g en and the initial output of pancreatic ju ce was determined During the following two 2 hr periods 180 ml of sterile sal ne v ith secret n were continuously infused a certain dose of sec etin be ng given during each per od These doses were 0 25 and 1 25 (exp 2) 0 5 and 1 0 (exp 3) 0 75 and 1 5 (exp 4) cl nical un ts per kg b w and hr

The pancreatic ju ce of t ansit onal stages i e the first half hour of each 2 hr per od was omitted

Results

Basal experiments (Table I) Mean volumes during the three 2 hr periods were 17 3 14 9 and 16 7 ml per 30 min (mean during the whole experiment 15 3) in dog A and 8 5 18 5 and 17 0 (mean 14 6) in dog B

Mean bicarbonate outputs were 0 175 0 278 and 0 281 meq per 30 min (mean during the experiment 0 245) in dog A and 0 109 0 199 and 0 157 (mean 0 155) in dog B

Secretin experiments Mean volumes during the initial periods were on the whole smaller than the volumes in the basal experiments (Table I) Mean bicarbonate outputs

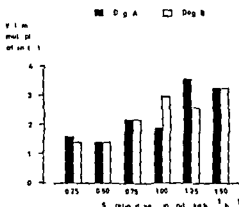


Fig 1

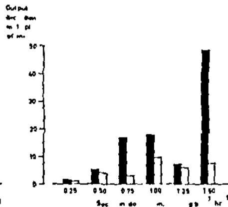


Fig 2

Fig 1 Volume of pancreatic juice during infusion of chemically pure secretin in two dogs
Fig 2 Output of bicarbonate during infusion of chemically pure secretin in two dogs

during the initial periods were smaller than the output in the basal experiment in dog A and equal or larger in dog B (Table I)

Mean volume during secretin infusion increased by increasing dose to 3 3 times the initial in both the dogs (Table II Fig 1) The bicarbonate output increased by increasing dose to 49 times the initial in dog A and 7 8 times in dog B (Table II Fig 2)

Discussion

The increase of the volume as well as the bicarbonate output was roughly proportional to the increase of the secretin dose The increase of the bicarbonate was more pronounced than that of the volume The increase of the volume at the dose 0 5 clinical units per hr and kg b w might be spontaneous as well as the increase of the bicarbonate output at 0 25 clinical units The largest increase of the bicarbonate output — nearly 50 times the initial — might depend on the low initial value during that experiment

A pancreatic duct fistula would be the most exact way to coll ct all the pancreatic juice It was shown by Sateri (1963) however that there is a fairly good agreement between the non surgical method used in the present study and a fistula method Thus it seems that doses without effect on the output of hepatic bile have a significant effect on the output and composition of pancreatic juice in the dog The effect may however be weaker than in man where a single dose of one unit gives a marked pancreatic response

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Acta physiol scand 1965 64 109—118

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Effect of Potassium on the Movement of Water Across the Isolated Amphibian Skin

By

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Abstract

Share L and H H Ussing *Effect of potassium on the movement of water across the isolated amphibian skin* Acta physiol scand 1965 *64* 109—118 — Substitution of potassium for sodium in the Ringer bathing the inner surface of the isolated toad skin resulted in an increased net movement of water across the skin along an osmotic gradient This effect was somewhat smaller than the response to vasopressin although the responses to the high potassium treatment and to vasopressin were not additive Substitution of sulfate for chloride as the major anion in the Ringer did not modify the relative increase in water movement following either the potassium or vasopressin treatments although the magnitude of the water movements were greater with the latter anion Low pH blocked the response to vasopressin but not to potassium Changes in the concentration of calcium in either inner or outer solutions were without effect on the responses to either potassium or vasopressin Parallel seasonal changes in the responsiveness of the toad skin to potassium and vasopressin were observed

Bricker Biber and Using (1963) reported that replacement of most of the sodium by potassium in the Ringer solution bathing the inner surface of the isolated frog skin resulted in a reduction in the transepithelial potential difference and a relatively smaller reduction in the short circuit current Vasopressin did not stimulate the short circuit current and the potential difference when the inner bathing solution was KCl Ringer although a stimulation was seen when the inner solution was K_2SO Ringer (Ussing Biber and Bricker 1964 unpublished observations) Consequently it was thought that it would be of interest to determine the effect of this high potassium treatment on the volume flow of water across the amphibian skin in the presence of an osmotic gradient A comparison of these effects with those of vasopressin has also been made

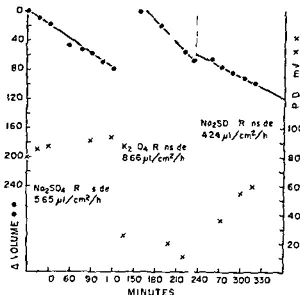


Fig 1 A representative experiment which shows the effects of substituting potassium for sodium in the inner solution on the transepithelial net water movement and potential difference The lines were fitted by inspection to the points relating change in volume of the outer solution to time The net water movements from the outer solution are calculated from the slopes of these lines The outer solut on was composed of 1/10 Na SO_4 Ringer

Methods

Frogs *Rana temporaria* of either sex or female toads *Bufo bufo* were doubly pithed A segment of abdominal skin d ssected free of muscle was mounted in the apparatus described by Koefoed Johnsen *et al* (1952) This apparatus permitted the measurement of the net movement of water from one side of the skin to the other and the potential difference across the skin The solution in contact with the inner surface of the skin (inner solution) was full strength Ringer 1/10 Ringer (outer solution) *i e* Ringer solution diluted 10-fold was in contact with the outer surface *The composition of the Ringer solutio is used has been given previously by* MacRobbie and Ussing (1961) In the high potassium periods of the experiments all of the sodium in the inner solution was replaced by potassium For the determination of the potential difference across the skin bridges containing 3 agar agar in Ringer led to saturated KCl solution which was in contact with calomel electrodes The potential difference was measured with a Radiometer PHM4 potentiometer The surface area of the skin was 7 cm in each experiment

An equilibration period of at least 45 min was allowed before measurements were begun There was usually an interval of 15 min between successive measurements The rate of net water movement from the outer solution to the inner solution was estimated graphically from the sl pe of the line relating the measured change in volume of the outer solut on to time (Fig 1 and 2) For this purpose 4 to 6 successive measurements which showed minimal scatter around this line were usually *required* although in a few cases only *3* measurements were used In experiments in which there were relatively large net movements of water both inner and outer solutions were changed between the d fferent treatment periods Within a few minutes after changing the inner solution from the usual Ringer in which sodium was the major cation (Na Ringer) to one in which potassium was the major cation (K Ringer) the outer soluti n usually began *to foam It was thought that this was due to secretion from the skin* glands stimulated by the elevated potassium concentration in the inner solution This was confirmed in a separate series of experiments in which the rate of secretion by the glands of the frog skin was measured (L Share and H H Ussing unpublished observations) Since this effect appeared to be completed within 20 min the outer s lution v as replaced by fresh 1/10 Na Ringer at the end of this interval and measurements of water movement were begun

Th initial experiments were performed on frogs which were brought into the laboratory in the fall and winter The responses to the high potass um treatment and to vasopress n were quite variable It was then found that uniform responses could be obtained with toads although the magnitude of the response varied w th the season and method of storing the animals Most

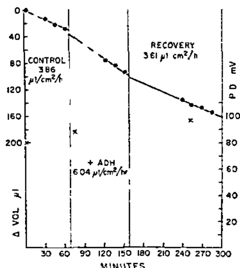


Fig 2 A representat ve experiment which shows the effects of the add tion of vasopressin to the inner solution on the trans epithelial net water movement and potential difference

of the e periments to be reported here were performed on toads wh h were brought into the laboratory in October 1962 and stored at 20 C for 2 to 3 months They were subsequently transferred to the cold room where they were kept at 4 C for several weeks prior to use The last experiments w th these toads which will be referred to as "Fall toads were completed by the end of April 1963 The toads used in the subsequent experiments Spring toads arri ed in the laboratory in April 1963 They were kept at 20 C without food until used

A commercial preparation of vasopress n (Ins p d n Alfred Benzon A/S Copenhagen) was employed It was added to the inner solution to a final concentration of 87 mU/ml Equ alent quant t es of vasopressin were added at approximately 45 min intervals to insure a maximal mainta ned effect of the hormone

In most of the experiments the degree of statistical significance was calculated by applying the t test to determine whether the d fferences between control and experimental periods were significantly different from zero In the experiments in which the conc ntrat on of calcium in the Ringer was varied analyses of covariance and variance were employed

Results

The replacement of all of the sodium in the inner solution by potassium resulted in an increase in net movement of water from outer to inner solutions In the Fall toads the volume flow of water was increased by about one third The relative response was independent of whether the major anion in the Ringer was sulfate or chloride (Table I A and C) although in chloride Ringer the absolute rates of water were approximately twice those in sulfate Ringer[1] In the Spring toads the response to potassium was considerably greater than in the Fall toads approximately three and one half times the control rate of water movement (Table V A and VI A) When the major anion in the Ringer solution was sulfate changing the inner solution back from K Ringer to Na Ringer resulted in an almost complete return of net water movement to control values (Table I A) Recovery in chloride Ringer was not as good (Table I C)

[1] The osmot c pressure of sulphate R nger is about 2 3 of that of chloride Ring

TABLE I Comparison of effects of potassium and vasopressin (ADH) in the inner solution on the net water movement ($\mu l/cm^2/hr$) across the toad skin The inner solution was Na Ringer in the control and recovery periods The outer solution was 1/10 Na Ringer in all periods Means ± S E The numbers in parentheses are the number of experiments Fall toads

I Control	II Experimental	III Recovery	II/I
A SO_4 ringer Na of inner solution replaced by K in experimental period			
6 19±0 51 (9)	8 38±0 57 (9)	6 25±0 68 (9)	1 36±0 09 (9)
B SO_4 ringer ADH in inner solution in experimental period			
4 95±0 44 (8)	7 58±0 57 (8)	4 99±0 42 (7)	1 61±0 17 (8)
C Cl ringer Na of inner solution replaced by K in experimental period			
10 68±0 95 (6)	14 24±1 36 (6)	13 10±1 13 (6)	1 32±0 04 (6)
D Cl ringer ADH in inner solution in experimental period			
10 71±1 29 (5)	16 00±1 83 (5)	11 65 (2)	1 54±0 20 (5)

Experimental period significantly different from control period at $p < 0.005$ < 0.05

TABLE II Comparison of effects of vasopressin (ADH) in Na SO Ringer and in K SO Ringer in inner chamber on net water movement ($\mu l/cm^2/hr$) across the toad skin Outer solution was 1/10 Na SO Ringer in all periods Means ± S E

I Na Ringer	II ADH in Na Ringer	III ADH in K Ringer	IV Na Ringer	II/I	III/II
A Fall toads 8 experiments					
5 79±0 72	7 58±0 42	8 24±0 69	6 19±0 88	1 50±0 27	1 08±0 04
B Spring toads 8 experiments					
10 8±1 6	40 8±7 4	42 1±7 8	21 0±3 6	3 99±0 73	1 08±0 16

[1] Significantly different from period I at $p < 0.01$

TABLE III Comparison of effects of K_2SO Ringer and K SO Ringer plus vasopressin (ADH) in inner chamber on net water movement (μl/cm /hr) across the toad skin. Outer solution was 1/10 Na SO Ringer in all periods Means ± S E Numbers in parentheses indicate number of experiments Fall toads

I	II	III	IV	II/I	III/II
Na Ringer	K Ringer	K Ringer + ADH	Na Ringer		
5 01±0 26 (6)	6 76±0 74 (6)	7 [illegible]4±0 [illegible]6 (6)	7 31±1 62 (4)	1 36±0 48 (6)	1 03±0 [illegible]6 (6)

TABLE IV Effect of low pH on response of toad skin to a high potassium concentration in inner solution. SO Ringer inside 1/10 Na SO Ringer outside 5 CO in air bubbled through inner and outer solutions during entire experiment Means ± S E. 7 experiments Fall toads

I	II	II/I
Na Ringer	K Ringer	
Net water movement μl/cm /hr		
4 41±0 39	5 34±0 62	1 19±0 08

Significantly different from I at p < 0 025

When the inner solution was changed from sodium to potassium Ringer there was a rapid fall in the potential difference across the skin (P D) Sufficient measurements were not made to determine this minimal P D with certainty There was then a slow small rise in P D to a peak which averaged 26 % of control in SO_4 Ringer and 17 % in Cl Ringer (Fall toads) The P D then fell gradually until the inner solution was changed back to Na Ringer In this recovery phase of the experiments the P D rose steadily although the P D had not returned to control levels after as long as two and one half hours The recovery P D averaged 65 % of control in Na_2SO_4 Ringer and 40 % of control in NaCl Ringer (Fall toads) when observations were discontinued one to two and one half hours after changing to Na Ringer These effects as well as the changes in water movement are illustrated by the representative experiment shown in Fig 1

The increase in net transepithelial water movement in response to the addition of vasopressin to the inside solution was consistently somewhat higher than the response to potassium *averaging 50 to 60 % in Fall toads and 300—460 % in Spring toads* (Table I B and D II B V B VI B Fig 2) As was the case with the response to potassium the relative response to vasopressin was essentially the same in both sulfate and chloride Ringer (Table I B and D) Recovery of water permeability following the wash

TABLE V Effect of changes in calcium concentration in inner solution on changes in net movement of water ($\mu l/cm^2/hr$) in response to high potassium and to vasopressin (ADH) in inner solution Cl Ringer inside 1/10 NaCl Ringer outside Means ± S E Spring toads

I Control	II Experimental	III Recovery	II/I
A Na of inner solution replaced by K in experimental period			
1 [Ca] = 0 6 experiments			
21.4±1.5	76.5±8.5	34.0±6.4	3.65±0.41
2 [Ca] = 0.9 mM 6 experiments			
18.5±2.1	58.1±3.5	27.4±3.0	3.45±0.42
3 [Ca] = 15 mM 5 experiments			
18.9±4.2	45.3±12.2	44.8±14.1	2.49±0.55
B ADH in inner solution in experimental period			
1 [Ca] = 0 6 experiments			
15.5±1.4	140±23	18.9±1.9	9.09±1.47
2 [Ca] = 0.9 mM 6 experiments			
23.6±3.9	33.4±16.7	28.4±3.9	4.40±0.90
3 [Ca] = 15 mM 6 experiments			
19.3±1.9	99.6±20.2	21.0±3.7	5.58±1.39

ing out of the vasopressin was almost complete under all conditions tested A representative experiment is shown in Fig 2

Two types of experiments were performed in order to determine whether the responses to vasopressin and the high potassium treatment are additive In one a period in which the inner solution was composed of Na_2SO_4 Ringer plus vasopressin was followed by a period with K_2SO_4 Ringer plus vasopressin as the inner solution (Table II) In the second type of experiment a period with K_2SO_4 Ringer inside was followed by one with K_2SO_4 Ringer plus vasopressin (Table III) The response to the combination of high potassium plus vasopressin was on the average 8 % greater than the response to either treatment alone However this difference was small and not statistically significant. Thus the two effects are not additive

It has been shown that reducing the pH of the inner solution to 6.5 completely blocks the action of vasopressin on the toad bladder (Bentley 1958 Schwartz *et al* 1960) The effect of low pH on the changes in net water movement of the toad skin in response

TABLE VI Effect of changes in calcium concentration in outer solution on changes in net movement of water (µl/cm²/hr) in response to high potassium and to vasopressin (ADH) in inner solution SO Ringer inside 1/10 Na SO Ringer outside Means ± S E Spring toads

I	II	III	II/I
Control	Experimental	Recovery	
Na of inner solution replaced by K in experimental period			
1 [Ca]=0 5 experiments			
9 13±0 55	31 8±6 6	19 3±6 0	3 52±0 75
2 [Ca]=10 mM 5 experiments			
7 65±0 95	25 4±4 8	12 1±1 8	3 4±0 69
B ADH in inner solution in experimental period			
1 [Ca]=0 5 experiments			
13 3 ±2 9	63 0±6 5	—	5 76±0 72
2 [Ca]=10 mM 6 experiments			
11 8 ±2 7	44 9±6 6	20 1±8 8	4 26±0 70

4 experiments

to high potassium treatment and to vasopressin was investigated Five per cent CO_2 in air was bubbled through both the inner and outer solutions reducing the pH in the inner solution to 6 3—6 5 and in the outer solution to 5 0—5 3 In 2 expts vasopressin added to the inner solution was without effect. The high potassium treatment increased the net movement of water across the toad skin by 19 % (Table IV) The magnitude of this response is approximately half that obtained at normal pH (Table I)

In the frog skin an increase in the calcium concentration in the inner solution resulted in a transient increase in short circuit current (Curran and Gill 1962) An increase in the calcium concentration of the outer solution reduced sodium permeability (Curran and Gill 1962) although the increment in sodium transport elicited by vasopressin was unaffected (Herrera and Curran 1963 Curran *et al* 1963) In the toad bladder there is evidence that calcium partially inhibits the action of vasopressin (Bentley 1959 Petersen and Edelman 1962) Bentley (1959) has also presented evidence that some calcium is required for the action of vasopressin upon this tissue For these reasons a study was made of the effects of changing the calcium concentration in both inner (Table V) and outer (Table VI) solutions on the responses to vasopressin and to the high potassium treatment In those experiments in which the concentration of calcium in the inner solution was varied the major anion was chloride The inner Ringer solutions containing calcium at either 0 mM (no added calcium) or 0 9 mM also contained sucrose

(45 mM and 42 4 mM respectively) so that the osmotic pressures of these solutions were equivalent to the Ringer containing 15 mM calcium The outer solution was the usual NaCl Ringer diluted ten fold and without added sucrose (final calcium concentration was 0 09 mM in all experiments) A change of the calcium concentration in the inner solution from 0 mM to 15 mM was without statistically significant effect on the responses to either potassium or vasopressin (Table V) The combination of high potassium and 15 mM calcium appeared to damage the skin irreversibly The potential difference across the skin fell much more rapidly than in those experiments in which the calcium concentration was 0 9 mM or 0 mM The change in net water movement was poorly reversible in the recovery periods when the inner solution was changed from K Ringer to Na Ringer the potential stabilized at — 5 to — 8 mV in four out of the 5 expts

Two experimental conditions were employed to study the effects of changes in the concentration of calcium in the outer solution zero calcium (no added calcium 0 5 mM sodium ethylene-diaminetetraacetate 20 mM sucrose) and 10 mM calcium There were no statistically significant differences in response to either potassium or vasopressin at these two calcium concentrations (Table VI)

It should be noted that changes in calcium concentration in either the inner or outer solutions were without effect on the water permeability of the toad skin under control conditions (Table V and VI) It was also found in both of these series of experiments that the response to potassium was significantly less than the response to vasopressin ($p < 0.05$ in the experiments in which the inner calcium concentration was varied $p < 0.025$ in the experiments in which the outer calcium concentration was varied)

Discussion

It appears reasonable to consider that the increased net water movement which occurred when potassium replaced the sodium in the inner solution was the result of an increased permeability of the toad skin to water However Reid (1892) demonstrated a slight net transfer of water across the frog skin when it is exposed to Ringer solution on both sides While it is possible that the high potassium treatment enhanced this phenonemon it appears to be unlikely

Certain aspects of the data which have been presented suggest the possibility that vasopressin and potassium act on some common mechanism to increase the permeability of the amphibian skin to water First the responses to these agents are not additive Second both agents act when in contact with the inner surface of the toad skin While it has been clearly demonstrated that vasopressin increases the permeability of a permeability barrier at the physiological outer surface of the amphibian skin and urinary bladder (MacRobbie and Ussing 1961 Hays and Leaf 1962) evidence relative to the locus of action of potassium is not available Third seasonal changes in the responsiveness of the amphibian skin to vasopressin are accompanied by parallel changes in the responsiveness to potassium

On the other hand a reduction in the pH of the inner solution completely blocks the action of vasopressin but at most only partially inhibits the response to potassium However it has been postulated that the first step in the action of vasopressin is its binding to the cell membrane (Rasmussen et al 1960 1963 Schwartz et al 1960) Evidence has been presented that an elevated hydrogen ion concentration may interfere

with this binding (Schwartz *et al* 1960) Thus it is possible that if vasopressin and an elevated potassium concentration trigger the same mechanism the potassium acts at some intermediate point the in sequence between the binding of the hormone to the membrane and the change in membrane permeability However the evidence for this concept is still highly circumstantial so that one must consider the alternative possibility that vasopressin and potassium increase the permeability of the toad skin to water by different mechanisms Should this be the case then it is however necessary to postulate that a high concentration of potassium in the inner solution inhibits the action of vasopressin since the two effects are not additive Some support for this possibility is found in the observation by Bentley (1959) that raising the concentration of potassium in the serosal medium to 10 mM decreases the water permeability of the isolated toad bladder in the presence of vasopressin Ussing Biber and Bricker (1964 unpublished observations) have shown that vasopressin does increase the short-circuit current in frog skins with K SO Ringer on the inside although there is no effect if the inner solution is KCl Ringer Thus potassium ions do not inhibit all effects of vasopressin It was rather suggestive that the increase in current induced by vasopressin in K SO treated frog skins was associated with a considerable swelling of the epithelium In the absence of vasopressin the epithelial volume remained constant for at least one hour if sulfate was the anion whereas there was spontaneous swelling and increase in short circuit current if chloride was the main anion One might therefore venture the hypothesis that high potassium increases the permeability of the outer diffusion barrier of the skin to water as well as to sodium by bringing about swelling of the epithelial cells The absence of a vasopressin effect on the current with KCl on the inside would be understandable if we assume that the swelling in KCl in itself gives near maximum effect on the sodium permeability If a similar explanation were valid for the effects on water permeability it would imply that the epithelial cells of toad skins swell in K_2SO_4 as well as in KCl Ringer This question is as yet open since the wrinkled surface of the toad skin makes it impossible to measure epithelial volumes according to MacRobbie and Ussing (1961) Quite generally osmotic swelling of the frog skin epithelium leads to increased active sodium transport and shrinkage to inhibition of the transport (Ussing 1964) If vasopressin increases the entry of sodium in the cells it would lead to swelling which in turn might also lead to increased water permeability

There is some disparity between the observations of Bentley (1959) and those presented here concerning the effects of changes in calcium concentration on the response to vasopressin Bentley found that a reduction in calcium concentration reduced the water permeability of the toad bladder in the presence of vasopressin in our experiments such an effect was not observed However Bentley omitted the calcium from the solutions bathing both sides of the bladder and there may also be qualitative differences in the responsiveness of the two tissues We have also failed to observe the diminution in response to vasopressin at elevated serosal calcium concentrations as reported by Bentley However the concentrations of vasopressin was 1 mU/ml in Bentley s experiments and 87 mU/ml in our experiments Petersen and Edelman (1962) reported that increasing the vasopressin concentration to 66—100 mU/ml abolished 8 % of the inhibitory effect of the elevated calcium concentration

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Monoamines in the Swimbladder of Gadus callarias and Salmo irideus

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Abstract

Fahlen G B Falck and E Rosengren *Monoamines in the swimbladder of Gadus callarias and Salmo irideus* Acta physiol scand 1965 64 119—126 —The noradrenaline in the swimbladder of the cod and the rainbow trout is stored in short adrenergic neurons whose perikarya are located in close relation to the swimbladder itself In the cod the noradrenergic neurons are innervated by noradrenergic neurons that arise from the coeliac ganglion of the sympathetic chain. A hitherto unknown system of epithelial cells storing 5-hydroxytryptamine was observed in the trout swimbladder

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Substantial evidence was presented by Fange (1953) that the muscular fibres of the secretory part of the cod swimbladder together with the radial muscle of the oval edge are sensitive to adrenaline (A) and noradrenaline (NA) Euler and Fange (1961) found that the muscularis mucosae and the gas gland in the swimbladder of the cod contained much greater amounts of NA than of A Indeed the amount of NA was of a magnitude that could well correspond to an adrenergic innervation of smooth musculature in the swimbladder However after denervation of the swimbladder no decrease of the NA content was observed, and it could not therefore be excluded that the NA in the swimbladder might be stored in places other than the adrenergic nerves presumably in some kind of chromaffin cells This assumption is interesting since it might imply a release of transmitter — in this case NA — from chromaffin cells. Such an organization was assumed to be a general principle in the adrenergic system by Burn and Rand (1960) and Brandon and Rand (1961) who suggested that NA is not localized in adrenergic neurons but in e g chromaffin cells This theory contradicts the reports of Euler's investigations (v Euler 1956 1961) which offer strong evidence that this amine is stored in the mammalian adrenergic neuron, principally in its terminal part The accuracy of Euler's concept was directly demonstrated recently by Falck and Torp (1962) and Falck (1962)

It is thus of considerable importance to obtain more accurate knowledge of the storage site of NA in the swimbladder This is now made possible by the introduction of a highly sensitive and specific fluorescence method for the detection of certain catechol amines and tryptamines at the cellular level (Falck and Torp 1961 Falck 1962 Falck et al 1962 Falck and Torp 1962)

Material and methods

The histochemical method is based on the principle that the catech lamines and 5-hydroxytryptamine (5-HT) can condense with formaldehyde to produce intensely fluorescent products in the tissues. Provided that the reaction takes place under fairly dry conditions no diffusion of the amines from their cellular localization will occur Usually the tissues to be analyzed must be freeze-dried Thin tissue sheets however can be dried for a short time at room temperature in vacuo usually without any diffusion of the amines. In fish swimbladder the last method may cause diffusion This however can be avoided by freeze-drying the stretch preparations of the tissue sheets

Gas glands of the swimbladder of the cod *Gadus callarias* including the distal part of nerves and vessels entering the swimbladder in the centre of the gas gland region, and also the coeliac ganglion and the splanchnic nerve were freeze-dried treated with formaldehyde gas for 1 hr at 80 C or 1 5 hrs at 60 C and then infiltrated with paraffin *in vacuo* Sections (8 μ) were mounted in Entellan (Merck) and examined under the fluorescence microscope in dark field illumination Pieces from different regions of the swimbladder of the rainbow trout *Salmo irideus* were treated in the same way

Thin sheets of the oval edge that separates the secretory and the resorbent parts of the swimbladder of the cod were carefully stretched over a plastic ring freeze-dried, and mounted in Entellan after a short time in xylene. The technique is described in detail elsewhere (Falck 1964 Falck and Owman 1964)

For the catecholamine and 5-HT analyses whole swimbladders of the rainbow trout were weighed and minced with 0 4 N perchloric acid. Dopamine (DA) NA and A were purified according to Bertler *et al* (1958) and determined as described by Häggendal (1963) 5-HT was estimated by the method of Bertler and Rosengren (1959) The coeliac ganglia and different parts of the swimbladder were treated in the same way The results are given in Table 1

Reserpine (Serpasil, Ciba) was given intraperitoneally to rainbow trout in doses of 5—10 mg kg body weight and day for a period of 4 days The animals were killed 5 days after the first injection

Results

Cod In the gas gland region and in the stretch preparations of the oval edge of the cod swimbladder a specific moderate green fluorescence developed in typical nerve bundles consisting of smooth fibres An intense green fluorescence developed in fine varicose fibres some of which were clearly seen to issue from the nerve bundles and thus obviously constituted the terminal parts of the neurons An abundance of varicose fibres was present in the gas gland and especially in the radial muscle of the oval edge numerous nerves also occurred in the rete mirabile and the muscularis of the secretory part and around the vessels In the wall of the resorbent part of the swimbladder the fluorescent fibres were much more sparse and seemed to be distributed to the vessels only No fluorescent cells were observed Nonfluorescent ganglion cells were found scattered at the base of the vascular bundles forming the rete mirabile The fine varicose green fluorescent fibres of the gas gland proper the rete mirabile and the muscularis were seen to originate from moderately green fluorescent ganglion cells situated immediately outside the swimbladder wall in the bundle of nerves and vessels



Fig. 1. Cod swimbladder. Section of the ganglion situated immediately outside the swimbladder wall in the bundle of vessels entering the organ in the centre of gas gland region. Moderately fluorescent ganglion cell bodies and their weakly fluorescent preterminal fibres are seen. Intensely fluorescent varicose fibres surround every ganglion cell body. Magnification 675.

entering the organ in the centre of the gas gland region. The smooth preterminal fibres from these ganglion cells were collected in a thick bundle accompanying the vessels into the wall. Beyond the entrance, this nerve bundle divided into several small branches. Some of them passed directly into the rete mirabile, the gas gland, and the muscularis of the secretory part, where they gave rise to the terminal varicose fibres. Other bundles accompanied the veins coming from the resorbent part to the oval edge.

The swimbladder is sympathetically innervated by branches from the right splanchnic nerve, which arises from the first 2 sympathetic ganglia. These ganglia often — as in the cod — constitute one single ganglion, called the *ganglion coeliacum* (Stannius, 1849). In the present investigation, this ganglion proved to be composed of relatively large ganglion cells emitting a weak green fluorescence; non-fluorescent cells could not with certainty be detected. Scattered among the ganglion cells was found a peculiar type of cells, irregular in form, that gave off short slender processes running in between a few of the contiguous nerve cells. These small cells exhibited a very strong green to yellow-green fluorescence, well comparable to that found in adrenal medullary cells. The same type of cells was found to occur in mammalian ganglia (Hamberger *et al.*, 1963; Falck *et al.*, 1964). The weakly fluorescent preterminal fibres from the ganglion

TABLE I Contents of NA (μg/g) in the swimbladder of the cod

Number of exp	Gas gland	Oval mucosa	The peripheral ganglion outside the gas gland
1	0 46	1 50	0 80
2	0.53	0 63	0 29
3	0 43	1 30	0 42

cells assembled to form the splanchnic nerve Along its course a small amount of scattered fluorescent ganglion cells was observed Most of the fibres in this nerve go to peripheral ganglia in the gut wall others are directed towards the swimbladder (Young 1931)

The last mentioned neurons transformed into highly fluorescent varicose fibres within the adrenergic ganglion situated contiguous to the swimbladder wall synaptically enclosing the fluorescent cell bodies in this ganglion (Fig 1)

The amount of NA in the different parts of the swimbladder is given in *Table I* The concentrations correlate well with the fluorescence microscopic findings except for the values found in the peripheral ganglion outside the gas gland Considering the number of nerve cells and the fluorescence intensity which was unusually high in the preterminal part of an adrenergic neuron, the concentration found seems to be too low However this can be explained by the fact that the ganglion could not be entirely freed from surrounding tissue In three determinations the coeliac ganglion was found to contain 7 6 μg g (4 7—9 2) catecholamine consisting mainly of NA It could not be excluded that a small proportion represented A It is in this connection interesting to note that the small highly fluorescent cells in mammalian ganglia may contain A (Owman and Sjostrand 1964) The NA values found seem to agree well with the histochemical findings and with the NA level in adrenergic ganglia found by other investigators (*cf* Muscholl and Vogt 1958)

Rainbow trout It seemed of great interest to study the adrenergic innervation apparatus also in a species with a swimbladder of the physostomous type that is with an open connection to the oesophagus and without a distinct gas gland The rainbow trout *Salmo irideus* was chosen for this purpose In the anterior part of the swimbladder which in the form of a narrow duct connects the organ with the oesophagus were observed a moderate number of nerve cells of varying size showing a rather weak green fluorescence The cells appeared in the connective tissue layer outside the muscularis scattered or in groups Some of the cells were situated in the vicinity of the muscle layer and in these instances the whole neuron could often be observed In such neurons the first part of the axon appeared smooth and rather weakly fluorescent but when the axon entered the muscle layer its character altered it became transformed into a varicose fibre that showed a much more intense fluorescence From the ganglion cell groups the axons traversed to the muscle layer collected in slender nerve bundles from which varicose fibres issued at the periphery of the muscularis A very considerable number of varicose fibres were found in the muscularis arranged parallel to the circular muscle fibres The occurrence of these fibres was extremely rich suggesting that every

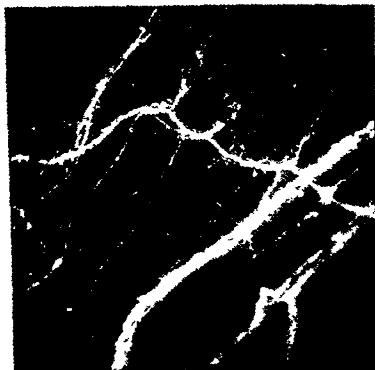


Fig. 7. Cod swimbladder. Stretch preparation of the oval edge. Adrenergic fibre bundles and varicose terminal fibres. Magnification 67×

muscle fibre made contact with one or more axons. Only occasional fibres passed into the subepithelial connective tissue layer. In other parts of the swimbladder the adrenergic innervation of the muscularis was the same as in the anterior part, but here no fluorescent ganglion cells were found.

The chemical analyses showed the presence of NA in a mean concentration of 0.33 μg/g (0.26—0.43) in the swimbladder of the rainbow trout. A and DA were not found in any significant amounts in that organ.

An interesting finding was that a yellow fluorescence developed in ovoid cells located in the epithelium of the swimbladder. These cells were generally provided with thin processes, one apical reaching the epithelial surface, and one or several reaching the base of the epithelium. These processes could be clearly observed because they also contained intensely fluorescent material. Their basal processes generally terminated in small enlargements. The fluorescent reaction in these cells suggests that they may contain a tryptamine. This agrees well with the results obtained from the chemical analyses which showed the presence of significant amounts of 5-HT (0.50 μg/g; 0.40—0.68).

After treatment with reserpine, no fluorescence or only a faint one was seen in the neurons, and only few weakly yellow fluorescent cells remained in the epithelium. The fluorimetric determinations revealed a considerable reduction of the 5-HT content (0.10—0.18 μg/g) and an almost complete depletion of NA.

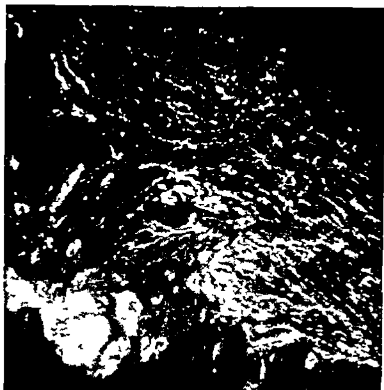


Fig 3 Rainbow trout swimbladder Transverse section of the wall of the anterior part demonstrating the abundance of varicose fibres in the circular muscle layer An adrenergic nerve cell body is seen in the surrounding connective tissue layer Magnification 750 ×

Discussion

The high sensitivity and specificity of the present method for the catecholamines and 5 HT are discussed at length by Falck (1962 1964) Falck *et al* (1962) and Corrodi and Hillarp (1963 1964) The reaction conditions under which the green fluorescence developed in neurons in the cod and trout tissue specimens and the properties of the fluorescent product indicate that a primary catecholamine is demonstrated Adrenaline containing neurons need a much longer exposure to formaldehyde gas to develop a maximal fluorescence (Falck *et al* 1963) Only cells that store huge amounts of A *i e* chromaffin cells in the adrenal medulla and in other locations show up under the reaction conditions used in this investigation *cf* Falck and Torp 1961 Dahl *et al* 1964 Owman and Sjostrand 1964 The only catecholamine found spectrophotofluorimetrically to be present in significant amounts was NA The histochemical criteria likewise indicate that the yellow fluorescent cells in the trout swimbladder epithelium contain a tryptamine derivative No other tissue structure developed this type of fluorescence Spectrophotofluorimetrically the trout swimbladder was found to contain significant amounts of 5-HT Furthermore after reserpine treatment a considerable decrease in

green and yellow fluorescence was observed and no NA and only slight traces of 5-HT could be found fluorimetrically. Thus there seems no doubt that the green neuronal fluorescence demonstrated NA and that the yellow fluorescence was derived from 5-HT stored in the special epithelial cells.

The morphology of the nervous structures in the swimbladders of the cod and trout was the same as in the adrenergic autonomous system of mammals (Hillarp 1959, Falck 1964). The distribution of the fluorescent material agrees well with the distribution of the adrenergic transmitter in mammalian adrenergic nerves (cf. Euler 1961, Falck 1962).

The finding of numerous adrenergic neurons located entirely peripherally explains well the result of the earlier denervation experiments. The possibility that the cod swimbladder contains chromaffin cells whose chromaffinity is due to a storage of catecholamines can be disregarded since no fluorescent cells — except ganglion cells — were found in the swimbladder and since the method used has a sensitivity of quite another magnitude than the chromaffin reaction.

The finding that the adrenergic neurons which arise in the coeliac ganglion store NA is surprising considering the fact that they terminate synaptically around the peripheral noradrenergic perikarya. Many investigations have demonstrated adrenergic fibres synaptically terminating upon adrenergic cell bodies in mammalian sympathetic ganglia (Falck 1962, Hamberger and Norberg 1963, Hamberger *et al.* 1963, Falck *et al.* 1964, Owman and Sjöstrand 1964) and there is support for the assumption that noradrenergic neurons are linked together in such ganglia (Hamberger *et al.* 1963).

The physiological significance of the 5-HT-containing cells in the trout swimbladder is not known. Morphologically these cells have the same appearance as the chromaffin cells in several mammalian species that also carry 5-HT and are provided with apical as well as basal processes (Falck, unpublished observations).

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Acta physiol scand 1965 64 127—135

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Occurrence of Dihydroxyphenylalanine Decarboxylase in Nerves of the Spinal Cord and Sympathetically Innervated Organs

By

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Recei ed 23 September 1964

Abstract

Anden N E T Magnusson and E Rosengren *Occurren e of dihyd oxyphenylalan ne decarboxylase in nerves of the spinal cord and sympa h tically innervated organs* Acta phys ol scand 1965 64 127—135 — After transection of the rabbit spinal cord the L 3 4-d hydroxyphenylalanine decar boxylase acti ity in the caudal part was reduced by 90—95 and 75—85 per cent when deter m ned *in vitro* and *in vivo* respectively The drop appeared to be fastest between the second and fifth day and the activity reached its minimum level after 3 weeks In the rabbit iris the di hydroxyphenylalanine decarboxylase activity fell to approximately 10 per cent of that of the control side in 48 hours after the excis on of the superior cervical ganglion In both tissues the time courses of the disappearance of dihydroxyphenylalanine decarboxylase activity and of noradrenal ne were very s milar but the amine disappeared somewhat more suddenly and rap dly than the enzyme Pregangl onic sympathectomy did not influence the dihydroxyphenyl alanine decarboxylase activity in the iris The nerve trunk contained only 15—20 times higher activ ty than the iris why the dihyd oxyphenylalanine decarboxylase like noradrenal ne prob ably occurs i i the whole neuron but is highly concentrated in the terminals Postganglion c sympathectomy reduced the dihydroxyphenylalanine decarboxylase activity in the rat sub maxillary gland and spleen by 85 and 80 per ent respectively

The enzyme L-3 4-dihydroxyphenylalanine (DOPA) decarboxylase was first demon strated in the guinea pig kidney (Holtz Heise and Ludtke 1938) It catalyzes the forma tion of dopamine (DA) from DOPA and is now generally accepted to be invol ed in the biosynthesis of catecholamines as was first proposed by Blaschko (1939) Later on strong evidences have been put forward for the view that the DOPA decarbox lase is identical with the L-5-hydroxytryptophan decarboxylase the corresponding enzyme in the biosynthesis of 5-hydroxytryptamine (5-HT) (Westermann Balzer and Knell 1958 Yuwiler Geller and Eiduson 1959 Bertler and Rosengren 1959 a Rosengren 1960) One of the the principal functions of the catecholamines and 5-HT in the bod seems to be serving as humoral transmitters of certain central as well as peripheral neurons The DOPA decarboxylase is however widely distributed n the organism

and actually most of the enzyme does not appear to be localized in neurons Until recently the relatively low activity of DOPA decarboxylase in the sympathetic ganglia and postganglionic trunks (Holtz and Westermann 1956) was the only evidence for the presence of the enzyme in nerves In a preliminary communication we have reported that the DOPA decarboxylase activity almost completely disappears from the spinal cord caudally to a transection and from the spleen after postganglionic sympathectomy (Andén Magnusson and Rosengren 1964) These findings prompted the present investigation into the time courses of the disappearance of the enzyme and noradrenaline (NA) after lesion of central as well as peripheral monoamine neurons

Experimental

Adult rabbits of either sex weighing 2—3 kg were used The spinal cord was transected at the level of Th 4—Th 6 This procedure like all other surgical operations in this investigation was carried out under pentobarbital sodium anesthesia Care was taken to prevent hypothermia residual urine decubitus and wound infections postoperatively In order to estimate the effect of the lesion on the DOPA decarboxylase activity *in vivo* the DA content of the cord was determined after administration of L DOPA (100 mg/kg i v) The animals were sacrificed by air embolism 60 min after the DOPA administration The spinal cord was carefully freed from the meninges and nerve roots The DOPA decarboxylase activity *in vitro* was determined in pieces of the thoracic cord 2 5—3 0 cm in length (0 2—0 6 g) cranially and caudally to the lesion The pieces were taken from the animals used for the DOPA decarboxylase determinations *in vivo* and were rapidly put into phosphate buffer instead of perchloric acid The extraction of the DOPA decarboxylase and the incubation with L DOPA were performed according to Bertler and Rosengren (1959 b) The DA formed *in vitro* as well as *in vivo* was estimated by the method of Carlsson and Waldeck (1958) as modified by Carlsson and Lindqvist (1962) The NA was determined as described by Bertler Carlsson and Rosengren (1958) Control values were obtained from animals not operated on

In other experiments the effect of sympathetic denervation on the DOPA decarboxylase activity in the rabbit iris was studied The left superior cervical ganglion and the continuous sympathetic chain cranially to the clavicle were removed After various intervals of time the animals were sacrificed by air embolism The DOPA decarboxylase activity of the right and the left iris was determined *in vitro* as described above The iris was preganglionically sympathectomized by excision of 3—4 cm of the left cervical sympathetic chain beginning about two cm caudally to the superior cervical ganglion

The DOPA decarboxylase activity in the denervated rat submaxillary and sublingual glands was also determined For reasons presented below it was necessary to ligate the ducts bilaterally when the enzyme activity in this tissue was to be determined The ligation was performed near the hilus On the same occasion the left superior cervical ganglion with the continuous sympathetic chain cranially to the clavicle were removed The operation was carried out under a Zeiss operation microscope The rats were sacrificed by a blow on the neck 14—16 days after the operation The right and left submaxillary glands including the sublingual glands were analysed separately for DOPA decarboxylase activity *in vitro* as described above The enzyme activity of the gland on the right side was used as a control value

The rat spleen was postganglionically sympathectomized by the excision of the celiac ganglion and as much as possible of the left and right splanchnic nerves as well as the soft tissue surrounding the celiac and superior mesenteric arteries A Zeiss operation microscope was employed After ten days or more the animals were decapitated The DOPA decarboxylase activity of the spleen *in vitro* was estimated as described above Control values were obtained from animals not operated on

Results

DOPA decarboxylase activity of the spinal cord The DOPA decarboxylase activity of the cord cranially to the section level was found to be 77 μg/g expressed as the DA formed *in vitro* after incubation with L DOPA for 45 min (S E 5 2 μg/g 26 expts) The enzyme

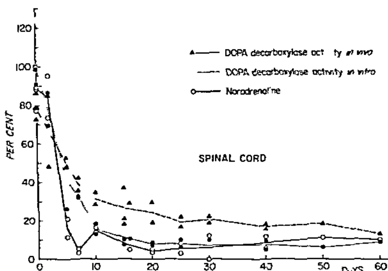


Fig 1 The dihydroxyphenylalanine (DOPA) decarboxylase activity and the noradrenaline concentration in the caudal part of the rabbit spinal cord at various intervals after transection Each symbol represents one single determination. *O dinate* enzyme activity and noradrenaline concentration in the part caudally to the lesion in per cent of that cranially

activity of this part of the cord in the operated animals did not differ significantly to that found in animals not operated upon As DA does not occur normally in measurable amounts in the spinal cord the DA formed *in vivo* after injection of L-DOPA can also be used as a measure of the DOPA decarboxylase activity The DA found in the part cranially to the transection 60 min after an i v injection of 100 mg/kg L-DOPA was 2 1 μg/g (S E 0 14 μg/g 26 expts) Nor did the values from the controls and the animals operated on differ significantly in these experiments The activities of the DOPA decarboxylase in the cord showed large individual differences when determined *in vitro* as well as *in vivo* It was found however that in the non-operated animal the activities were about the same in pieces taken cranially and caudally to the transection (see Fig 1) As the transection had no apparent effect on the activity cranially to the lesion the activity of the DOPA decarboxylase in the caudal part is given as per cent of that found in the cranial part It was found that transection caused a considerable reduction of the DOPA decarboxylase activity in the caudal part of the cord as determined *in vitro* as well as *in vivo* (Fig 1) The drop of the DOPA decarboxylase activity was most marked between the second and fifth day but a gradual reduction of the enzyme activity was found to occur during the following two weeks After that time the enzyme activity was not further reduced it reached a constant level corresponding to 5—10 and 15—25 per cent of the cranial one as determined *in vitro* and in *vivo* respectively

The NA dropped in the caudal part of the spinal cord from section Fig 1 as described earlier (Andén *et al* 1964 The NA level cranially to the lesion was 0 26 μg g (S E 0 018 μg g 24 expts 60 min after the injection of L-DOPA 100 mg kg i v) There was no significant change of the NA content in this part of the cord after opera

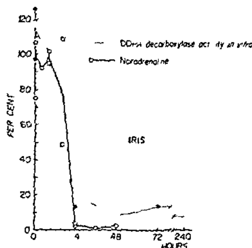


Fig 2 The dihydroxyphenylalanine (DOPA) decarboxylase activity *in vitro* and the noradrenaline content in the rabbit irides at various intervals after the excision of the superior cervical ganglion Each symbol represents one single determination *Ordinate* DOPA decarboxylase activity of the denervated iris in per cent of that of the innervated one and noradrenaline content of the denervated irides in per cent of that of the innervated ones

tion Caudally the NA concentration was approximately the same as that found in the cranial portion of the cord of the non operated animals and seemed unchanged during the first 2 postoperative days During the following three days the NA level showed a steep drop and after 7 days the NA had almost completely disappeared in the caudal part

DOPA decarboxylase activity of the iris The DOPA decarboxylase activity in the iris of the right side (i e of the side not operated upon) was 5 5 μg/iris (S E 0 47 μg iris 14 experiments) expressed as the amount of DA formed *in vitro* after incubation with L DOPA for 45 min Between 8 and 16 hrs after the excision of the left superior cervical ganglion the decarboxylase activity of the left iris substantially dropped (Fig 2) The minimum level was reached after about 48 hrs when only approximately 10 per cent of the original activity was observed The values of the NA in the iris after postganglionic sympathectomy (Fig 2) are taken from a previous investigation (Andén *et al* 1964) Sixteen hours after the operation almost all of the NA remained while during the following 8 hrs it disappeared almost completely During the NA disappearance there was a distinct mydriasis on the sympathectomized side

The decarboxylase activity in the iris ten days after preganglionic sympathectomy on the left side was assayed in two experiments The activities of the left iris were 97 and 94 per cent of those of the right one

The postganglionic nerve trunks immediately adjacent to the superior cervical ganglia were analyzed for their DOPA decarboxylase activity in 2 expts on normal rabbits and compared to those found in the irides and the ganglia of the same animals The DA formation *in vitro* in the nerve trunk extracts was 860 and 620 μg/g while it was 57 and 33 μg g in the iris extracts i e the decarboxylase activity was 15—20 times higher at the former site The DOPA decarboxylase activity in the superior cervical ganglia of these animals was 4 900 and 5 600 μg/g i e 5—10 times higher than that in the postganglionic trunks

DOPA decarboxylase activity of the rat submaxillary gland It was not possible to detect any DOPA decarboxylase *in vitro* in the normal submaxillary gland It was also found

TABLE I The dihydroxyphenylalanine (DOPA) decarboxylase activity of the rat submaxillary plus sublingual glands The enzyme activity was determined in vitro and is expressed as μg dopamine formed per g tissue after incubation with L DOPA for 45 min The left superior cervical ganglion was removed 14—16 days earlier On the same occasion the ducts of the glands were ligated bilaterally

	Number of experiments	DOPA decarboxylase activity (mean ± S E)	Difference
Right (=control) side	5	101 ± 14 7	$p < 0.001$
Left (=denervated) side	5	16 ± 3 0	

TABLE II The dihydroxyphenylalanine (DOPA) decarboxylase activity of the rat spleen The enzyme activity was determined in vitro and is expressed as μg dopamine formed per g tissue after incubation with L DOPA for 45 min In the denervated group the celiac ganglion was excised 10—46 days earlier Nonoperated rats were used as controls

	Number of experiments	DOPA decarboxylase activity (mean ± S E)	Difference
Controls	6	8 2 ± 0 96	$p < 0.001$
Denervated	6	1 7 ± 0 09	

that no DOPA decarboxylase activity of a brain extract which if incubated alone showed a rather high DA formation could be demonstrated if it was incubated together with an extract from an intact submaxillary gland However there was a clearcut DOPA decarboxylase activity in extracts from submaxillary glands which had atrophied in consequence of duct ligation about 14 days earlier (Table I) Further there was no reduction of the DOPA decarboxylase activity in an extract from brain when incubated together with an extract from a gland duct ligated After the excision of the superior cervical ganglion the DOPA decarboxylase activity in the duct ligated gland was reduced by some 85 per cent about 14 days later (Table I) The corresponding NA loss amounted to more than 95 per cent

DOPA decarboxylase activity of the rat spleen The DOPA decarboxylase activity was determined in vitro in extracts from the rat spleen 10—14 days after the excision of the celiac ganglion The operation caused a drop of the enzyme activity by on the average 80 per cent (Table II) The NA level was reduced by 80—95 per cent after the same operation

Discussion

It is probable that the DOPA decarboxylase is present in monoamine nerves both in the central nervous system and in the periphery as after the degeneration of these nerves the organs studied in this investigation showed an almost complete disappearance of this enzyme activity The functional disturbance which occurs after cutting the nerve cannot be the cause as preganglionic sympathectomy of the iris did not produce the same effect To our knowledge the present investigation is the first to show that an enzyme participating in the metabolism of catecholamines and 5 HT is present at the mentioned sites Since long nerve degeneration experiments have indicated that the NA in the peripheral organs is localized in sympathetic nerve endings (Euler and Purkhold 1951 Goodall 1951) This view has been found to be true by means of the fluorescence method for histochemical demonstration of certain catecholamines and tryptamines (Falck 1962) Recently investigations using this technique combined with biochemical studies and transection experiments have proved that the NA and 5 HT of the spinal cord are situated in descending nerve tracts (Carlsson *et al* 1964 Magnusson and Rosengren 1963 Carlsson Magnusson and Rosengren 1963) There is however a marked difference in the time course of the disappearance of the NA in a peripheral organ and that of the monoamines in the spinal cord after denervation The depletion of the NA from the rabbit iris is completed within 24 hrs after the removal of the superior cervical ganglion whereas the drop of the NA and 5 HT in the rabbit spinal cord occurred 2—7 days after the transection (Andén *et al* 1964) It is noteworthy that the disappearance of the DOPA decarboxylase from the spinal cord and the peripheral organs after denervation apparently showed a similar difference the enzyme activity in the iris had reached its minimum within 48 hours whereas at the same interval of time it was on the whole unchanged in the spinal cord The disappearance of DOPA decarboxylase and NA followed roughly similar time courses after denervation but the enzyme did not seem to vanish as rapidly and suddenly as the amine The relatively high value of the enzyme activity in the caudal part of the spinal cord five days after transection may be explained by the apparently somewhat slower degeneration of the axons belonging to the 5 HT than to the NA neurons (Andén *et al* 1964) However in the spinal cord it was evident that the last part of the enzyme was lost much later than NA and 5 HT which may at least partly be due to the barrier between the central nervous system and the blood

Also after the section of cholinergic nerves the amine (acetylcholine) and the enzyme responsible for its formation (choline acetylase) seem to disappear from the innervated structures Hitherto only the cholinergic nerve terminals in the sympathetic ganglia have been investigated in this respect The choline acetylase activity of the superior cervical ganglion falls almost to zero after removal of part of the cervical sympathetic chain (Feldberg 1943 Banister and Scrase 1950 Hebb and Waites 1956) The time course of the loss of the choline acetylase activity largely parallelled that of acetylcholine found by Mac Intosh (1938) and Feldberg (1943) The losses of acetylcholine and choline acetylase from the superior cervical ganglia appear to occur at a rate which lays between the corresponding rates in the spinal cord and the iris with respect to the disappearance of NA and DOPA decarboxylase

There is strong evidence for the view that NA occurs everywhere in the adrenergic postganglionic neurons but that it is highly concentrated in the terminals present in the organs (Euler 1956 p 136 1959 Falck 1962) The same conditions seem to prevail in

the central neurons storing NA DA and 5 HT (Dahlstrom and Fuxe 1964) as well as in the peripheral neurons containing acetylcholine and choline acetylase (for references see Hebb 1963) As mentioned above the DOPA decarboxylase in the nervous system was first detected in the non terminal parts of the postganglionic sympathetic neurons (Holtz and Westermann 1956) Our finding that there is a factor of only 15—20 between the enzyme activity in the nerve trunk and in the iris indicates that the decarboxylase has a similar intraneuronal distribution as NA This statement is based on the assumption that the sympathetic nerves in the iris like in the spleen amount to not more than 1/1 000—1/10 000 of the organ weight (Euler 1956 p 136) The somewhat higher decarboxylase activity in the superior cervical ganglia than in the postganglionic trunks may at least partly be due to the presence of noradrenergic nerve terminals at the former site recently discovered (Hamberger and Norberg 1964)

The figures obtained for the DOPA decarboxylase activity in the caudal part of the spinal cord after transection were found to be lower if the determination was performed *in vitro* than *in vivo* The reason for the difference may possibly be that part of the DA found in the spinal cord after DOPA administration was formed outside the central nervous system and brought there by the blood and accumulated in the vessel lumen or in the vasomotor nerves This alternative does not seem very likely however as the pia membrane always was carefully removed and it contains most of the large vessels and the sympathetic nerves (Carlsson *et al* 1964) It is more probable that the difference is due to the DOPA decarboxylase in the capillary walls of the central nervous system recently described (Bertler Falck and Rosengren 1964) The activity in the capillary walls appears to be negligible compared with that in the descending nerves to judge from results obtained *in vitro* But the capillary decarboxylase might be of greater importance in the enzyme assays carried out *in vivo* than *in vitro* since all the L-DOPA in this case must first pass through the capillary walls before reaching the decarboxylase containing axons

The finding that DOPA decarboxylase in the submaxillary glands could be demonstrated only after ligation of their ducts may be due to an enzymatically break down of the decarboxylase in an extract of an intact gland It has been observed that duct ligation produces a reduction of the gland activity of *i e* the protease and amylase (Junqueira 1951) The duct ligation does not diminish the NA content but gives a pronounced concentration of the adrenergic ground plexus due to the atrophy of the parenchyma cells (Andén Norberg and Olsson 1964) After this operation there is an accumulation of mast cells and the 5-hydroxytryptophan decarboxylase in them may contribute to the small discrepancy between the minimal levels of the NA content and the DOPA decarboxylase activity obtained after postganglionic sympathectomy Likewise the mast cells in the rat spleen may be responsible for the similar phenomenon observed in this organ after excision of the celiac ganglion Nor in the rabbit iris did the DOPA decarboxylase reach the same low level as NA after postganglionic sympathectomy but the agreement was better than in the rat tissues A great deal of the apparent DOPA decarboxylase activity in the iris salivary gland and spleen after postganglionic sympathectomy may however be due to the non-enzymatic decarboxylation as well as the errors involved in the determination of small amounts of DA This assumption is strengthened by our finding that after heat denaturation (100 C, 10 min) of an iris extract the value obtained for the DA formation was no less that about 10 per cent of of the control value *i e* of about the same magnitude as after postganglionic sympathectomy

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Acta physiol scand 1965 64 136—143

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The Urinary Excretion and the Tissue Uptake of Noradrenaline after Severe Tissue Depletion of Noradrenaline by α-Methyl-Meta-Tyrosine and Metaraminol

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Abstract

Andén, N E *The urinary excretion and the tissue uptake of noradrenaline after severe tissue depletion of noradrenaline by α methyl meta tyrosine and metaraminol* Acta physiol scand 1965 64 136—143 Four to eleven hours after an i v injection of metaraminol (0 2 mg/kg) to rats pre treated with α-methyl *m* tyrosine (400 mg/kg intraperitoneally on each of the two preceding days) there was a severe reduction of the noradrenaline levels in the sympathetically innervated organs but no significant change in urinary noradrenaline excretion This finding may indicate that the release of noradrenaline from the sympathetic nerves is unaffected in spite of severe transmitter depletion The uptake of tritiated noradrenaline from the blood by the sympathetically innervated organs was about 40 per cent of normal after this treatment Metaraminol (and its precursor α methyl *m* tyrosine) is almost as efficient as reserpine in causing depletion of the adrenergic transmitter but this effect appears to be due to simple displacement rather than interference with transmitter storage and release

During the last few years a great deal of attention has been paid to α methyl *m* tyrosine (αMMT) on account of its ability to produce a severe depletion of noradrenaline (NA) from central and peripheral neurons The active agent is in fact the metabolite metaraminol (α methyl β hydroxy *m* tyramine) Also after injection of this drug there is a drop of the NA in sympathetically innervated organs (for references see Carlsson 1964 a Andén 1964 The missing NA is almost stoichiometrically displaced by metaraminol both after administration of this substance and of αMMT (Carlsson and Lindqvist 1962 Carlsson 1964 a Andén 1964 Udenfriend and Zaltzman Nirenberg 1964 Shore and Alpers 1964 In an earlier study we succeeded in getting displacement of about 95 per cent of the NA in sympathetically innervated organs of rats and cats Not even after this intense treatment was it possible to detect impairment of the sympathetic nerve function (Andén and Magnusson 1964) It is to be noted that (—) metaraminol

is about 3 times weaker in biological activity than (—) noradrenaline This finding has prompted investigations into other aspects of the NA metabolism than the tissue levels after such treatment In this paper results are reported from rat experiments on the NA excretion in the urine and the tissue uptake of labelled NA from the blood after severe displacement of the tissue bound NA by metaraminol

Material and methods

Adult hooded rats of both sexes we ghing 200—350 g were used throughout Since we wanted to study the urinary excretion of the NA from the sympathetic nerves only adrenal demedulla tion was carried out by enucleation b laterally under pentobarbital sodium anesthesia (Farris and Griffith 1949 p 444) During the week after the operation the animals had access to 0 9 per cent sod um chloride solution in addition to tap water The demedullat on appeared satisf ctory as the adrenals in the few cases examined always were almost completely depleted of catecholamines when determined according to Bertler Carlsson and Rosengren (1958) further there was practically no adrenaline (A) excret on in the urine (see results) At least 2 weeks elapsed between the adrenal demedullation and the collect on of the control urine

The excretion of NA and A in the urine from the same groups of three rats was assayed before and after severe tissue depletion of NA by αMMT plus metaraminol In both cases the urine was collected for 7 hrs During this time the rats were restrained in separate wire cages The hindlegs were pulled through holes of the cage floors The feet were fixed to the cage stands by means of adhes e plaster The urine was vo ded directly into tubes each containing 1 ml N perchloric ac d The tubes were fixed to the stands between the feet The an mals had free access to tap water and all of them were given about 10 ml 0 9 per cent sod um chloride solut on i p divided into sev ral doses The high diuresis thus maintained might reduce such sources of error as an incomplete emptying of the bladder as well as an oxidation of catecholamines in a non acid bladder urine Miction was always provoked at the beginning and at the end of the urine collection The urines from the 3 rats were pooled The tubes were washed with 3 ml N per chloric acid which were added to the urine specimen

The urine specimen was divided into two equal aliquots They were treated in parallel through the whole procedure after the addition of 5 μg NA and 5 μg A to one of them The free catecholamines were purified accord ng to Euler and Lishajko (1959) by passing the urine sample (pH 8 2) through a column of alum nium oxide and by eluting with acetic acid D fferen tial estimation of NA and A was performed spectrophotofluorometrically as described by Bertler Carlsson and Rosengren (1958) The recove ies of 5 μg were on the average 57 per cent for NA and 70 per cent for A All values were corrected for the recovery

At least one week after the collect on of the control urine the rats were treated with αMMT plus metaraminol as reported earlier (Andén and Magnusson 1964) On 2 consecutive days αMMT was g ven intraper toneally in a dose of each 400 mg/kg (DL form monohydrate) On the third day 0.2 mg/kg laevo-metaram nol (as L-(+) bitartrate) was injected into a femoral vein The urines from the treated rats were collected between 4 and 11 hrs after the admin stra t on of the metaraminol Afterwards the animals were mmediately sacrificed by decap tat on and some org ns removed for the determ nation of the NA levels The animals used as controls of the NA levels as well as those sacrificed 4 hrs after the metaram nol injection w e allowed to move freely This was true also for some rats sacrificed after 11 hrs (see results) The NA contents of the heart spleen and femoral muscles were est mated spectrophotofluorometrically fter ion exchange chromatography and ox dat on as described by Bertler Ca lsson and Rosengren (1958)

The uptake by the heart and femoral muscles of labelled NA from the blood was studied n rats non pretreated as well a rats depleted of NA as described above In both cases (±) NA H in a dose of 1 0 / g/kg b w w s injected into a ta l ve n The (±) NA 7 H HCl w th a specific ctı ıty of 5 8 C/mmole was obtained from New England Nucl r Corporation Pri r to use this m terial was shown to be pure by paper chromatography In th p treated rats the NA H was given 3 hrs and 45 min after the injection of the m taraminol All an mals were sacrificed by decapitation 30 m n after the nject on of the NA H Th hearts and femoral muscles were removed as fast as poss bl Their contents of NA H w e d term ned in a l qu d scintillation counter after ion e hange chrom tography (Carlsson n l Waldeck 1963)

TABLE I The concentrations of noradrenaline (μg/g mean ± S E) in different tissues of rats treated with α methyl *m* tyrosine plus metaraminol

	Heart	Spleen	Femoral muscles
1 No treatment	0.74±0.051 (n=8)	0.42±0.044 (n=5)	0.097±0.0021 (n=4)
2 4 hrs after metaraminol	0.06±0.015 (n=3)	0.03±0.009 (n=4)	0.005±0.0023 (n=4)
3 11 hrs after metaraminol without urine collection	0.08±0.013 (n=3)	0.02±0.003 (n=3)	0.005±0.0025 (n=3)
4 11 hrs after metaraminol with urine collection 4 hrs 11 hrs	0.18±0.014 (n=8)	0.18±0.010 (n=8)	0.012±0.0027 (n=3)
	4–1 p<0.001 4–3 p<0.01	4–1 p<0.001 4–3 p<0.001	4–1 p<0.001 4–3 0.1<p<0.2

TABLE II The effect of combined treatment with α methyl *m* tyrosine and metaraminol on the urinary excretion of noradrenaline (NA) and adrenaline (A) in adrenal demedullated rats

	NA in μg/kg hr		A in μg/kg hr	
	Control period	Treatment period	Control period	Treatment period
Group 1	0.46	0.33	0.10	0.12
Group 2	0.46	0.44	0.05	0.06
Group 3	0.44	0.70	0.07	0.03
Group 4	0.50	0.62	0.00	0.02
Group 5	0.67	0.49	0.00	0.09
Mean±S E	0.51 ±0.042	0.52±0.065	0.04±0.013	0.06±0.019

Results

The tissue levels of NA After treatment with αMMT (400mg/kg i p on each of the two *preceding days) plus metaraminol* (0.2 mg/kg i v) there was a considerable reduction of the NA concentrations in the sympathetically innervated organs of rats (Table I). The heart spleen and femoral muscles were depleted of 90—95 per cent of their original NA content 4 hrs after the administration of the metaraminol The NA levels did not seem to change during the following 7 hrs in the animals not restrained and fixed Unexpectedly there was a rise of the NA during the same time in the rats from which the urines were collected This phenomenon was apparently most significant in the spleen where the NA concentration increased from about 5 to about 40 per cent of normal

TABLE III The concentrations of tritiated noradrenaline (ng/g mean ± S E) in the heart and femoral muscles of control rats and rats treated with *a* methyl *m* tyrosine plus metaraminol The rats were injected with tritiated noradrenaline (1 0 μg/kg i v) 30 min before sacrifice

	Heart	Femoral muscles
Controls	3 6±0 22 (n=5)	0 118±0 0099 (n=5)
Treated	1 5±0 08 (n=6)	0 041±0 0083 (n=6)
	p < 0 001	p < 0 001

The urinary excretion of NA The values of the NA excretion in the urine obtained before and after the tissue depletion of NA are presented in Table II In the control experiments before the displacement of the tissue bound NA by metaraminol the adrenal demedullated rats were found to excrete NA to the urine on the average 0 51 μg/hr and kg b w The A excretion was 0 04 μg/hr and kg b w *i e* the adrenal demedullation appeared successful (In a few preliminary experiments it was observed that without adrenal demedullation the A and NA excretions were about equal and that this operation changed the NA excretion little) At least one week after the collection of the control urine the same groups of three rats were treated with αMMT (400 mg/kg i p on each of the 2 days preceding the urine collection) and metaraminol (0 2 mg/kg i v 4 hrs before the start of the urine collection) Between 4 and 11 hrs after the administration of the metaraminol the rats excreted on the average 0 52 μg NA and 0 06 μg A per hr and kg b w

The tissue uptake of NA ^3H After a severe displacement of the tissue bound NA with metaraminol there was a significant reduction of the tissue uptake of labelled NA from the blood (Table III) In the control rats the heart and femoral muscles contained 3 6 and 0 118 ng/g (ng = 10 g) NA ^3H respectively 30 min after an i v injection of 1 0 μg per kg b w NA ^3H The same dose was given i v 3 hrs and 45 min after metaraminol (0 2 mg/kg i v) to rats pretreated with αMMT (400 mg/kg i p on each of the 2 preceding days) At sacrifice 30 min later there was 1 5 and 0 041 ng/g NA ^3H in the heart and femoral muscles respectively *i e* 42 and 35 per cent of the control values

Discussion

Like reserpine αMMT plus metaraminol can produce an almost complete depletion of the tissue NA After reserpine treatment stimulation of the sympathetic nerves fails to evoke the normal functional response (Carlsson *et al* 1957 Muscholl and Vogt 1958) This reserpine effect is probably due to an inhibited release of NA from the sympathetic nerve endings since the output of NA to the blood obtained in untreated animals at intense ganglionic stimulation by carbacholine is almost completely blocked after reserpine treatment (Bertler *et al* 1958) Reserpine also causes a considerable drop in the urinary NA of rabbits (Carlsson *et al* 1957) rats (Leduc 1961 Johnson 1963) and humans (Gaddum Kwiatkowski and Laverty 1958 Carlsson Boje Rasmussen and

Kristjansen 1959) These urinary findings support the view that the NA in the urine mainly originates in postganglionic sympathetic nerves (Euler 1956 p 288) Therefore the apparently unchanged urinary excretion of NA from the adrenal demedullated rats after severe displacement of the tissue bound NA by metaraminol may indicate that there is no reduction of the NA release from the sympathetic nerve endings after this treatment This interpretation of the results obtained in the present study gives an explanation of our previous observation that this treatment does not impair the sympathetic nerve function although at least 90 per cent of the NA in the tissues is displaced by the biologically about 3 times less active metaraminol (Andén and Magnusson 1964)

The urinary excretion of NA was 2—7 times higher in the present investigation than in earlier ones where the urine was collected from rats placed in metabolic cages (Schapiro 1958 Crawford and Law 1958 Höjfelt and Byjdeman 1961 Perman 1961 Leduc 1961 Gunne 1963 Johnson 1963) The discrepancy may partly be due to the fact that the urine in this study was discharged directly into an acid solution By this procedure we avoided such sources of error as catecholamine oxidation on the way to the collection bottle or drying of the urine on the walls There are however also other explanations of the high NA excretion It has been observed that a subcutaneous injection of a small volume of saline to rats increases the urinary excretion of NA (Crawford and Law 1958) an effect probably not caused by increased diuresis (Euler 1956 p 167) and in all our experiments about 10 ml of saline was given i p The restrained and fixed rats frequently struggled to get free during the collection period Increased muscular work has been shown to produce a substantial rise of the NA output to the urine (Euler and Hellner 1952) It should not be a disadvantage that the impulse flow of the sympathetic nerves was high in our urine experiments since we wanted to know if the NA release from the nerves was affected or not It has been found that at cold stress when the sympathetic tone is high (Leduc 1961) the NA excretion to the urine after reserpine treatment is more diminished relatively to untreated controls than at room temperature (Johnson 1963)

It is not unlikely that an increased impulse flow of the sympathetic nerves also was the cause of the significant rise of the NA levels in the animals used for the urine collection A high sympathetic tone should produce an increased release of the metaraminol from the store and simultaneously an increased synthesis of NA (Euler and Hellner Björkman 1955) It is likely that the release of metaraminol from the nerve endings was of an order of magnitude much smaller than that of NA Part of the newly synthesized NA was probably taken up in the store replacing the lost metaraminol The NA levels did not rise very rapidly however and were significantly depressed compared with the untreated controls also at the end of the urine collection i e the urinary output of NA appeared unchanged despite severely depleted tissue stores of NA during the whole period

Reserpine blocks almost completely the uptake of NA from the blood by the sympathetic nerves of peripheral organs (Muscholl 1960 Hertting Axelrod and Whitby 1961) There is a certain temporal correlation between NA uptake and adrenergic nerve function after reserpine treatment the uptake is almost completely inhibited as long as the adrenergic transmission is blocked when the nerve function recovers the uptake rapidly rises to about 40 per cent of that of untreated controls (Andén Magnusson and Waldeck 1964) The tissue levels of NA are very low at the time of recovery of the nerve function and return to normal much more slowly than the uptake mechan

ism It is noteworthy that in the present investigation the same discrepancy between NA uptake and NA concentration existed after treatment with αMMT plus metaraminol Hess *et al* (1961) reported that αMMT to guinea pigs reduced the uptake of labelled NA by the heart also by some 60 per cent but in their experiments the NA drop was only about 70 per cent It seems thus as the adrenergic transmission is unimpaired at a NA uptake about 40 per cent of the normal since this value was obtained both after treatment with αMMT plus metaraminol and at the recovery from the sympathetic blockade produced by reserpine As mentioned above at both instances the sympathetic nerves function apparently unimpaired although the sympathetic innervated organs are depleted of at least 90 per cent of their NA From these data it appears that only a small fraction of the NA in store is immediately necessary for nerve function Hillarp (1960) was the first to discover the non homogeneous nature of the adrenergic store by showing that the catecholamines in the adrenal medullary granules occur in two pools one large stable fraction bound to an equivalent amount of adenosine phosphates and one small labile fraction bound in an unknown manner Monoamines seem to be primarily incorporated into the small fraction both in adrenal medullary and nerve granules and it is probable that the amine release takes place to a normal extent only when this mechanism is intact (for references see Carlsson 1964 b) There are several possibilities of the difference between the uptake of NA ^{3}H in the animals untreated and those given αMMT plus metaraminol or those in the recovery phase after reserpinization For example in the non pretreated animals approximately 60 per cent of the NA ^{3}H may be accumulated in the large functionally rather inert fraction and the incorporation into this fraction may be blocked for a long time after treatment with αMMT plus metaraminol or with reserpine Anyhow even after the severe depletion of the tissue NA by αMMT plus metaraminol the sympathetic nerves can incorporate a considerable amount of NA ^{3}H and this ability may explain why the NA release and thus the adrenergic transmission is apparently unimpaired

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Properties of Afferent Connections to the Rostral Spinocerebellar Tract in the Cat

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Abstract

Oscarsson O and N Uddenberg *Properties of afferent connections to the rostral spinocerebellar tract in the cat* Acta physiol scand 1965 *64* 143—153 — Properties of the rostral spinocerebellar tract (RSCT) have been studied and compared with those of its functional hindlimb equivalent the ventral spinocerebellar tract (VSCT) Both RSCT and VSCT are monosynaptically activated from high threshold Group I muscle afferents and polysynaptically influenced from the flexor reflex afferents Excitation from Group I afferents is mono- di and polysynaptic in the RSCT but exclusively monosynaptic in the VSCT The monosynaptic linkage has different properties in the two tracts The effects from the flexor reflex afferents are predominantly excitatory in the RSCT and predominantly inhibitory in the VSCT The significance of similarities and differences in the organization of the two tracts is discussed

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The rostral spinocerebellar tract (RSCT) is activated from ipsilateral forelimb nerves It is anatomically distinct from the dorsal spinocerebellar tract (DSCT) in arising from cell bodies rostral to Clarke s column and in occupying a relatively ventral position in the cord and from the ventral spinocerebellar tract (VSCT) in being uncrossed In addition the RSCT differs from these tracts in reaching the cerebellum through the restiform body as well as the brachium conjunctivum (Oscarsson and Uddenberg 1964)

The DSCT and VSCT have no components related to the forelimbs and front part of the body (Holmqvist Oscarsson and Uddenberg 1963b The RSCT is however a functional equivalent of the VSCT This is demonstrated by the general similarity in the organization of afferent connections and the similar mode of termination in the cerebellar cortex Oscarsson 1964b Oscarsson and Uddenberg 1964 Both RSCT and VSCT are monosynaptically activated from high threshold Group I muscle afferents and polysynaptically influenced from the flexor reflex afferents FRA

This paper describes the properties of the synaptic linkage between Group I afferents and RSCT neurones The characteristics of the FRA connections to these neurones will also be reported. The significance of similarities and differences in the organization of the RSCT and VSCT will be discussed

Methods

The experiments were done on cats The body temperature was kept between 36 and 38.5 C. The blood pressure was continuously recorded and prevented from falling below 80 to 90 mm Hg Artificial ventilation was used in most experiments.

a Mass discharge recording The animals were usually under pentobarbitone anaesthesia In the left forelimb the following nerves were dissected and mounted for stimulation the muscle component of the suprascapular nerve (SSC) the axillar nerve (Ax) the nerve to the long head of the triceps (LHT) the biceps nerve (B) the deep radial nerve (DR) the superficial radial nerve (SR) the median nerve (Med) and the ulnar nerve (Uln) The radial nerve was prepared in the right forelimb and the hamstring triceps surae and superficial peroneal nerves were prepared bilaterally in the hindlimbs The spinal cord was exposed through a laminectomy including the second and third cervical vertebrae. Mass discharges in the RSCT VSCT and DSCT were recorded from the left lateral funiculus dissected and mounted on recording electrodes as described previously (Laporte Lundberg and Oscarsson 1956 Holmqvist *et al* 1963 b) The dorsal funiculus was dissected at the same segmental level to permit monophasic recording of primary afferent volleys Volleys from the hindlimb nerves were monitored by triphasic recording from a proximal part of the sciatic nerve.

b Unit recording The animals were unanaesthetized and either decerebrated or decapitated They were paralysed with gallamine The forelimb nerves dissected for stimulation were those listed above The right hamstring nerve was prepared for stimulation in order to permit identification of VSCT units otherwise the hindlimbs were left intact. The left dorsolateral surface of the cord was exposed through a small opening in the caudal part of the lamina of the second cervical vertebra The dura was split and the cord lightly pressed against the roof of the spinal canal by pellets of gel foam inserted beneath the cord outside the dura This procedure together with bilateral pneumothorax, prevented respiratory and circulatory movements allowing stable recording with capillary microelectrodes from fibres in the lateral funiculus The afferent volleys were recorded triphasically from the dorsal funiculus at the same segmental level (C3)

"*Ia*" and "*Ib* afferents" are used synonymously with large muscle spindle and tendon organ afferents "*Ipsilateral*" and "*contralateral*" refer to the side of the ascending axons *Stimulus strength* is expressed in multiples of the strength necessary for evoking a barely visible ingoing volley

Results

A comparison between the RSCT and VSCT is of considerable interest as these two tracts related to the forelimbs and hindlimbs respectively can be regarded as functional equivalents Most of the experiments were designed to permit recording from both tracts in the same animal Identification of the tracts was based on the finding that ascending spinal neurones activated monosynaptically from Group I muscle afferents belong to the RSCT when related to the ipsilateral forelimb and to the VSCT when related to the contralateral hindlimb (Lundberg and Oscarsson 1962 Oscarsson and Uddenberg 1964) The VSCT has previously been studied in considerable detail (Oscarsson 1956 1957 1960 Eccles Hubbard and Oscarsson 1961a, Lundberg and Oscarsson 1962) and our observations on this tract are in agreement with those already reported.

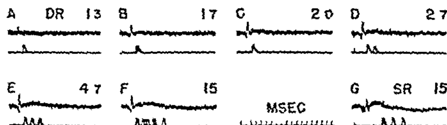


Fig 1 Recording from RSCT unit (lower trace) on stimulation of the deep radial nerve (DR records A—F) and the superficial radial nerve (SR record G) at indicated strengths The afferent volley (upper trace) was recorded triphasically from the dorsal funiculus at the same segmental level as the microelectrode (C3) Two or three superposed traces

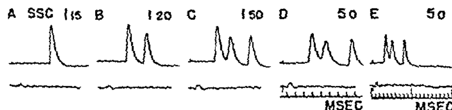


Fig 2 Recording from RSCT unit (upper trace) on stimulation of the muscle component of the suprascapular nerve (SSC) at indicated strengths. The afferent volley (lower trace) was recorded triphasically from the dorsal funiculus at the same segmental level as the microelectrode (C3) Records D and E were obtained simultaneously at different speeds

1 Connections with Group I afferents

a Synaptic linkage The response evoked in RSCT neurones by a volley in Group I afferents has been studied by recording from more than 110 units in 15 expts The number of connections investigated is larger as most of the RSCT neurones received Group I excitation from more than one nerve The response was usually a single spike with a short latency indicating a monosynaptic linkage For example in Fig 1 stimulation of the deep radial nerve evoked an impulse with a latency of 1 0 msec as measured relative to the afferent volley recorded at the same segmental level This value corresponds approximately to the synaptic delay as the conduction velocity is about the same in the primary afferents ascending in the dorsal funiculus and in the tract fibres (Rexed and Ström 1952 Oscarsson and Uddenberg 1964

Observations made on some RSCT units indicate that di and polysynaptic excitation from Group I afferents also occurs In occasional units a spike appeared after a latency of more than 2 msec at a low strength This latency decreased abruptly to 1 msec or less at higher strengths of stimulation A unit with this behaviour was illustrated in a previous paper (Oscarsson and Uddenberg 1964 Fig 1 In other units the latency was long also on stimulation at maximal Group I strength The unit shown in Fig was activated by volleys in the suprascapular nerve At a low strength producing only a small afferent volley A an action potential appeared after a latency of 4 0 msec With a large Group I volley the latency decreased to 2 3 msec (B C Units of this

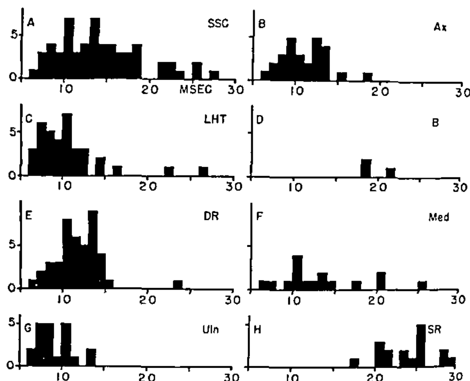


Fig 3 Latency of first spike evoked in RSCT units by a volley in Group I afferents (A—G) and cutaneous afferents (H) Ordinate number of cases Abscissa latency of first spike relative to *afferent volley recorded at the same segmental level as the unit response (C3) The following* nerves were stimulated the muscle component of the suprascapular nerve (SSC) the axillar nerve (Ax) the nerve to the long head of triceps (LHT) the nerve to biceps (B) the deep radial nerve (DR) the median nerve (Med) the ulnar nerve (Uln) and the superficial radial nerve (SR) Histograms A—E sample spikes evoked by Group I volleys that were less than 70 per cent maximal and histograms F and G spikes evoked at stimulation strengths of less than 1 3 times threshold See the text

type were usually activated by Group I afferents in other nerves after a latency proving a monosynaptic linkage and can hence be identified as RSCT neurones

Action potentials evoked after a latency indicating a di or polysynaptic linkage were often observed on stimulation of the suprascapular nerve but occurred also on stimulation of Group I afferents in the other nerves tested as shown by the histograms in Fig 3 A—G It should be noted that the discharge evoked by a volley in the biceps nerve in each of the 3 cases observed had a latency indicating intercalation of at least one interneurone (Fig 3 D) Histograms A—E sample spikes evoked by Group I volleys that were less than 70 per cent maximal This would exclude any appreciable contribution from Group II afferents in most cases Histograms F and G relating to the mixed median and ulnar nerves were obtained by sampling responses evoked at stimulus strengths below 1 3 times threshold which would exclude contribution from cutaneous afferents and Group II muscle afferents in the majority of the cases

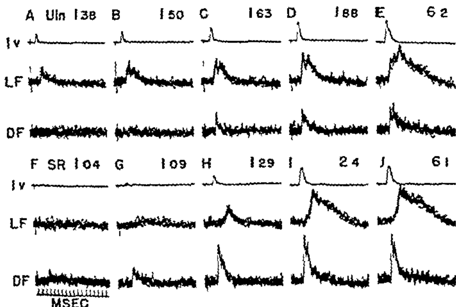


Fig 4 Experiment to demonstrate threshold separation between Group I afferents and cutaneous afferents in the ulnar nerve The traces show from above downwards afferent volley in the dorsal funiculus (I v) mass discharge in tracts ascending in the intermediate one third of the lateral funiculus (lateral fascicle LF) and mass discharge in tracts ascending in the dorsal one third of the lateral funiculus (dorsal fascicle DF) The dorsal funiculus and the dorsal and ventral fascicles were dissected and mounted for recording at the C3 level A—E stimulation of the ulnar nerve (Uln) and F—J stimulation of the superficial radial nerve (SR) at indicated strengths

The threshold separation between Group I afferents and cutaneous afferents in the ulnar nerve is illustrated in Fig 4 The mass discharge evoked in a lateral fascicle containing the RSCT was compared with the mass discharge evoked in a dorsal fascicle containing the dorsomedial cutaneous tract which presumably is identical with the spinocervical tract (Holmqvist *et al* 1963 b) On stimulation of the ulnar nerve the RSCT discharge was appreciable at 1 38 (A) and large at 1 50 times threshold (B) The discharge in the cutaneous tract appeared at 1 5 times threshold (B) There was little need for spatial summation in the cutaneous tract as illustrated by records F—J (lower traces) obtained on stimulation of the superficial radial nerve Hence it can be concluded that few cutaneous afferents were included in the volley from the ulnar nerve before the stimulus strength had been raised above 1 5 times threshold Similar observations were made in other experiments on stimulation of the median and ulnar nerves and showed that few if any cutaneous afferents were excited by stimuli below 1 3 times threshold

The RSCT unit shown in Fig 3 responded with a repetitive discharge to stimulation of Group I afferents in the suprascapular nerve (B C) The low threshold argues against the possibility that the later impulses were produced by Group II afferents and so does the absence of further spikes at higher strengths with activation of many Group II afferents (D E) A repetitive discharge produced by a Group I volley was relatively often observed on stimulation of the suprascapular nerve The initial spike had either a short monosynaptic or long latency Repetitive activity was only exceptionally

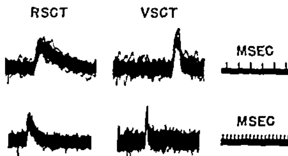


Fig 5 Configuration of mass discharge in RSCT and VSCT as recorded from the lateral funiculus dissected in the third cervical segment The discharges were evoked by slightly submaximal Group I volleys from the ipsilateral deep radial nerve (RSCT) and the contralateral hamstring nerve (VSCT) The upper and lower records were obtained simultaneously at different speeds

evoked by Group I volleys in the other nerves (*cf* Oscarsson 1965a Table I) Presumably the repetitive discharge was due to prolonged asynchronous bombardment from interneurones

The records in Fig 5 show, on a fast (upper traces) and slow time base the mass discharges in the RSCT and VSCT evoked by volleys in Group I afferents and recorded from the lateral funiculus dissected in the upper cervical region The difference in configuration of the discharges is conspicuous The long duration of the RSCT discharge is due to the late activity evoked in the RSCT units and described above The duration was long already when the discharge first appeared on stimulation of muscle nerves at 1 2—1 4 times threshold strength and did not change markedly with additional activation of more Group I afferents (Holmqvist *et al* 1963b Fig 2 Q—T) This is in agreement with the observations made on single fibres and suggests that the early and late spikes in the RSCT units were evoked by the same category of high threshold Group I afferents The short duration of the mass discharge in the VSCT (Fig 5) is explained by the brief EPSPs (excitatory post synaptic potentials) which are evoked in the tract neurones through their exclusively monosynaptic connections with Group I afferents (Eccles *et al* 1961a)

The front of the discharge in the RSCT was less steep than that in the VSCT (Fig 5) The reason for this difference is obscure The front is formed by the monosynaptically elicited spikes in the individual neurones The Group I volley from forelimb nerves (Holmqvist *et al* 1963b Fig 2 A—H) is as synchronous as that from hindlimb nerves and it seems unlikely that unequal conduction velocities of the RSCT fibres would introduce any significant temporal scatter considering the short conduction distance in the RSCT (about 4 5 cm) Perhaps the primary afferent collaterals leading to the RSCT neurones have widely different conduction velocities and therefore activate these neurones after varying delays

b Synaptic properties It has previously been shown that the properties of the synaptic linkage between Group I afferents and VSCT neurones in some respects differ from those of the synaptic linkage between Group I afferents and DSCT neurones (Eccles *et al* 1961 a Eccles Oscarsson and Willis 1961 b) The observations described below demonstrate differences in the properties of the monosynaptic linkage to RSCT and VSCT neurones respectively

The findings in Fig 6 and 7 are typical of a series of experiments concerned with changes in the synaptic transmission during and after repetitive stimulation In Fig 6 the amplitude of the mass discharge in the RSCT VSCT and DSCT has been plotted

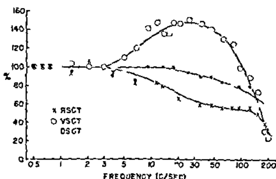


Fig 6 Effect of frequency on amplitude of mass discharge in the RSCT VSCT and DSCT The amplitude as percentage of the slowest frequency response is plotted against frequency of stimulation The mass discharges were recorded from the lateral funiculus dissected and mounted for stimulation at the C3 level The discharge in the RSCT was evoked by combined stimulation of the ipsilateral deep radial nerve and the ipsilateral nerve to the long head of triceps the discharge in the VSCT by combined stimulation of the contralateral hamstring and triceps surae nerves and the discharge in the DSCT by combined stimulation of the corresponding ipsilateral nerves

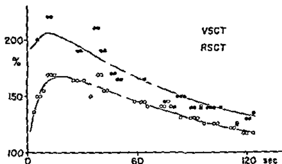


Fig 7 Post tetanic potentiation of mass discharge in RSCT and VSCT Stimulating and recording arrangement as in Fig 6 Ordinate amplitude of mass discharge as percentage of value obtained before the tetanus Abscissa time after tetanus (duration one minute frequency 300 stimuli per second) The testing discharges were elicited at a frequency of 0 55 per second

against stimulation frequency The measurements were made after a steady value had been reached after about 10 stimuli at the low frequencies and after a much larger number of stimuli at the high frequencies The mass discharge in the VSCT increased at frequencies above 3—5 per sec became maximal at 10—50 per sec and decreased below the control value at frequencies above 100 per sec The increase of the VSCT discharge on repetitive stimulation is due to a corresponding increase in the size of the Ib EPSPs in VSCT neurones (Eccles *et al* 1961 a) On the other hand the RSCT discharge decreased in amplitude at frequencies above 3 per sec This suggests that the EPSPs in RSCT neurones decrease in size on repetitive stimulation just as the Ia EPSPs in limb motoneurones (Curtis and Eccles 1960) and the Ia and Ib EPSPs in DSCT neurones (Eccles *et al* 1961b) The DSCT discharge did not decrease until the rate of the stimulation was increased above 15—20 per sec whereas the EPSPs in DSCT neurones start to decrease in size already at frequencies above 1 per sec (Eccles *et al* 1961b) Presumably the resistance of the DSCT discharge at higher frequencies was due to a large safety margin of the synaptic linkage (cf Oscarsson 1956b)

Complementary information was obtained by recording from single units Most RSCT and VSCT neurones followed presynaptic stimulation rates of several hundreds per sec initially After a period of stimulation (several seconds) the highest frequency

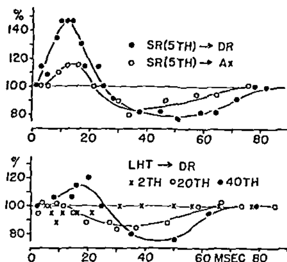


Fig 8 Effects on RSCT mass discharge produced by conditioning volleys in cutaneous and muscle afferents Upper graph the conditioning volley was elicited in the superficial radial nerve (SR) and the testing volley in the deep radial (DR) and axillar nerve (Ax) respectively Lower graph the conditioning volley was elicited in the nerve to the long head of triceps (LHT) and the testing volley in the deep radial nerve The conditioning volleys were elicited at the stimulation strengths indicated on the graphs The amplitude of the conditioned discharge in per cent of the unconditioned one is plotted against volley interval in msec

that was followed faithfully had decreased to between 100 and 200 per sec in most RSCT and VSCT neurones

Previous investigations have demonstrated that the post tetanic potentiation of the mass discharge in the VSCT is much larger than in the DSCT though smaller than the potentiation of the monosynaptic reflex (Oscarsson 1957) The potentiation following a prolonged tetanus is preceded by a depression which has a shorter duration in the VSCT and DSCT than in the monosynaptic reflex (Lloyd 1949 Oscarsson 1957) It has now been found that the post tetanic potentiation in the RSCT resembles that in the other two spinocerebellar tracts in having an initial depression of short duration (Fig 7) This depression was somewhat more conspicuous in the RSCT than in the VSCT (Fig 7) In the three experiments performed the potentiation was less in the RSCT than in the VSCT (Fig 7) though it was larger than the potentiation usually found in the DSCT (Holmqvist Lundberg and Oscarsson 1956 Oscarsson 1957 McIntyre and Mark 1960)

2 Connections with FRA

The effects produced in RSCT neurones by volleys in cutaneous and high threshold muscle afferents were studied on mass discharge and unit recording A conditioning cutaneous volley always facilitated the RSCT mass discharge irrespective of whether the testing discharge was evoked from a muscle nerve innervating structures close to or distant from those innervated by the cutaneous nerve (Fig 8 upper graph) A slight depression usually followed the facilitation

Volleys in high threshold muscle afferents were less effective There was usually a weak facilitation followed by some depression but sometimes only a weak (less than 15 per cent) facilitation or inhibition In the example shown in Fig 8 (lower graph) a conditioning volley in Group I afferents (crosses) produced no appreciable effect whereas additional activation of Group II and III muscle afferents produced some inhibition (open circles) and at higher strengths of stimulation also some initial facilitation (filled circles)

A volley in FRA afferents often elicited a discharge in the RSCT neurones. For example in Fig. 1 stimulation of high threshold (Group II and III) muscle afferents (D—F) and cutaneous afferents (G) produced repetitive activity. The latency of the first impulse elicited from the skin nerve and measured relative to the afferent volley recorded at the same segmental level, varied from 1.7 to many msec. The shortest latencies are sampled in the histogram of Fig. 3 H. The first spike was usually evoked at a low stimulus strength and the latencies indicate that the transmission was di- or polysynaptic. The minimal latency of the spikes evoked from high threshold muscle afferents was 1.4 msec longer than the shortest latency of the spikes evoked from Group I afferents. This latency is too long to be explained alone by the slower conduction velocity in Group II afferents. It suggests that the synaptic linkage was di- or polysynaptic.

Discussion

The RSCT is a forelimb equivalent of the VSCT. This is demonstrated by its mode of termination in the cerebellum which is similar to the termination of the VSCT and characteristically different from the termination of the DSCT and the cuneocerebellar tract (Oscarsson and Uddenberg 1964). It is also demonstrated by the general similarity in the organization of the afferent connections to the RSCT and VSCT. Both tracts are monosynaptically activated from high threshold muscle afferents, which can be identified as tendon organ afferents (Oscarsson 1956, 1957, 1960, 1965a; Eccles *et al.* 1961a; Holmqvist *et al.* 1963b), and polysynaptically influenced from the FRA. Furthermore, the convergence of Group I excitation to individual neurones is similar in the RSCT and VSCT. In both tracts the convergence is extensive, each neurone being usually activated from synergic muscle groups at several joints, sometimes even from one muscle group at each of the main joints of the limb (Oscarsson 1957, 1965a; Eccles *et al.* 1961a).

However, there are also conspicuous differences in the organization of the two tracts. Group I afferents activate RSCT neurones not only through a monosynaptic linkage but also through di- and polysynaptic connections. On the other hand, the linkage between Group I afferents and VSCT neurones is exclusively monosynaptic (Eccles *et al.* 1961a). The monosynaptic linkage has different properties in the two tracts: in the RSCT transmission is depressed on repetitive stimulation at frequencies between 5 and 100 per sec, whereas it is facilitated in the VSCT. This is presumably due to corresponding changes in the size of the EPSPs that are elicited in the tract neurones. The time course and magnitude of the post tetanic potentiation were also different in the RSCT and VSCT. The significance of the differences in synaptic properties is unknown; they presumably represent adaptations to special functions. Differences of the same order have been observed in the properties of the synaptic connections of other functionally related pathways. For example, the Ia EPSPs in limb motoneurones decrease in size on repetitive stimulation, whereas they increase in respiratory motoneurones of the thoracic cord (Curtis and Eccles 1960; Sears 1964).

The FRA effects are predominantly excitatory in the RSCT and predominantly inhibitory in the VSCT. This difference between the two tracts may be of little general significance as it is known that some VSCT neurones in the cat receive predominantly excitation from the FRA (Oscarsson 1957) and that excitation is the dominant effect in the VSCT of the dog (Oscarsson, Rosén and Uddenberg 1964).

In addition to these functional distinctions between the RSCT and VSCT there is one major anatomical difference the RSCT is uncrossed and the VSCT crossed at the segmental level The importance of this fact is apparent when related to the recent disclosure that uncrossed and crossed ascending spinal tracts differ in fundamental respects (Oscarsson 1956 1964a Magni and Oscarsson 1962 Holmqvist and Oscarsson 1963 Szentagothai 1964) The uncrossed tracts ascend in the dorsal part of the lateral funiculus and originate from cell bodies in the dorsal or dorsomedial part of the spinal grey matter The crossed tracts ascend in the ventral part of the lateral funiculus and in the ventral funiculus and originate from cell bodies in the ventral or ventrolateral part of the grey matter Furthermore the uncrossed tracts have ipsilateral receptive fields of polysynaptic actions whereas the crossed tracts have bilateral fields In accordance with the latter "rule" it has been found that the polysynaptic effects from the FRA are ipsilateral in the RSCT and bilateral in the VSCT (Oscarsson 1957 1965a)

The fact that the RSCT belongs to the uncrossed tracts and the VSCT to the crossed tracts suggests that they have arisen independently from different embryological anlages by parallel evolution of similar patterns of organization The two tracts would be analougous rather than serially homologous structures The reason why the hind limb and forelimb tracts have developed as separate anatomical entities is unknown A similar situation is encountered in connection with the DSCT and the cuneocerebellar tract These two pathways relate to the hindlimbs and forelimbs respectively and contain largely equivalent channels for proprioceptive and exteroceptive information (Holmqvist Oscarsson and Rosén 1963a) On the other hand there is no evidence for anatomical differences between the forelimb and hindlimb components of the major pathways to the cerebrum the dorsal funiculus-medial lemniscus system and the spinocervical tract (Kruger Siminoff and Witkovsky 1961 Andersson 1962 Oswaldo-Cruz and Kidd 1964) though it has recently been shown that the former system contains a forelimb but not a hindlimb channel for information from large muscle spindle afferents (Oscarsson and Rosén 1963)

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From the Institute of Physiology University of Lund Sweden

Integrative Organization of the Rostral Spinocerebellar Tract in the Cat

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Abstract

Oscarsson O *Integrative organization of the rostral spinocerebellar tract in the cat* Acta physiol scand. 1965 64 154—166 — The convergence of synaptic actions from Group I muscle afferents and flexor reflex afferents (FRA) to rostral spinocerebellar tract (RSCT) neurones, and the effects produced in these neurones by natural stimulation of receptors have been investigated The monosynaptic excitation from Group I afferents was related mainly or exclusively to tendon organ afferents The convergence of Group I excitation to individual tract neurones was usually extensive there was often excitation from one muscle group at each of the shoulder elbow and wrist and finger joints The RSCT neurones received polysynaptic excitation from the FRA of large receptive fields in the ipsilateral forelimb These fields had no obvious relation to the Group I receptive fields The effects evoked by stimulation of cutaneous receptors were weak The FRA effects from muscle were often strong on maintained stretch there was only moderate adaptation and cessation of stretch was followed by an afterdischarge *The organization in the RSCT and VSCT (ventral spinocerebellar tract) is compared* and the information forwarded by the two tracts is discussed — Additional observations demonstrated that VSCT neurones were influenced from the FRA not only in hindlimb but also in forelimb nerves. There is a discussion of the significance of this finding for a hypothesis concerning the informative value of the FRA connections to ascending tracts.

The course and termination of the rostral spinocerebellar tract (RSCT) and the general organization of the afferent connections to this tract were described in previous papers (Oscarsson and Uddenberg 1964 1965 The RSCT is activated monosynaptically from high threshold Group I afferents and polysynaptically from the flexor reflex afferents FRA The RSCT is a functional equivalent of the ventral spinocerebellar tract (VSCT) as demonstrated by the similar mode of termination in the cerebellar cortex and the similar organization of afferent connections (Oscarsson 1964b Oscarsson and Uddenberg 1964 The receptive fields of the RSCT neurones are described in this paper together with observations on natural stimulation of receptors The findings permit a further comparison of corresponding features in the RSCT and

VSCT The experiments have been designed to permit recordin from both these tracts in the same animal and some new observations on the VSCT have been added to tho e describ d previously (Oscarsson 1956 1957 1960 Eccles Hubbard and Oscarsson 1961 Lundberg and Oscarsson 1962)

Some of the findings have been reported in brief (Oscarsson 1964b)

Methods

The technique and the p eparations have been described in prev ous papers (Oscarsson and Uddenbe g 1964 1965) Electrophys ological identifi ation of RSCT and VSCT neurones has been based on the finding that ascending spinal neurones activated monosynapti ally from Group I muscle afferents belong to the RSCT when related to the ips lateral fo elimb and to the VSCT when related to the contralateral h ndl mb (Lundberg and Oscarsson 1962 Oscarsson and Uddenberg 1964)

In the ipsilateral forel mb some or all of the following nerves were prepared for stimulat on the muscle component of the sup ascapular nerve (SSC) the ax llar nerve (Ax) the ner e to the long head of triceps (LHT) the nerve to biceps (B) the deep radial nerve (DR) the median nerve (Med) the ulnar nerve (Uln) and the superficial radial nerve (SR) The latter three or four nerves were mounted on stimulati g electrodes but usually left n connect on ith the periphery in order to permit stimulation of receptors Nerves not dissected for stimulation were left intact except a few (the musculocutaneus the cutaneus medialis and ome muscles branches in the upper arm) which were cut du ing the dissection The rad al nerve was dissected for stimulati n in the contralateral forel mb wh ch was otherwise left intact The contralateral hamstring nerve was the only nerve dissected and mounted for sti nulation in the h nd l mbs

Some experiments were designed to study effects on natural stimulation of muscle receptors The ipsilateral forelimb was denervated except for the deep radial a d median nerves which were mounted on stimulati g elect odes and left in connection ith the periphery The tendons of the follow ng muscles ere prepared for pulling by hand or load ng extensor carpi rad alis extenso d gitorum communis extensor d gitorum lateralis extensor carpi ulnar s flexor profundus d gitorum palmaris longus and flexor carp radialis Gallamine as g ven in order to protect the nerves from damage due to muscle contract o s

Ips lateral" and *contr lateral*" refer to the side of the ascend ng axons *Sti ulu stren th* is g ven n mult ples of the strength necessary for evoking a barely vis ble ingo ng olley

Results

1 *Patterns of convergence*

a Group I afferents The contribution from G oup I afferents in various nerves to the activity in the RSCT was studied by recording the mass discharge in the tract In Fig 1 the intermediate one third of the lateral funi ulus was d ssected for recordin in the upper cervical cord This part of the funiculus contains the RSCT but not the dorsomedial cutaneous tract ith its prominent monosynaptic discharge evoked from cutaneous afferents (Holmqvist Oscarsson and Uddenberg 1963b) The RSCT discharge evoked by a maximal Group I volley in 5 muscle nerves is shown by records A—E Stimulation of the suprascapular axillar and deep radial nerves produced large discharges the triceps (LHT) nerve a small discharge and the biceps nerve a hardl vis ble discharge Th mixed median and ulnar nerves were stimulated at 2 5 times threshold strength which would activate cutaneous afferents and Group II muscle afferents in addition to the Group I affe ents Oscarsson and Uddenber 1965 Fig 4) Stimulation of the skin nerve only evoked a mall early response (H in this pa t of the funiculus It can be concluded that the init al monos naptic component of the mass di charges evoked from

TABLE I Convergence of excitatory action to 40 RSCT units in 3 experiments (I—III) Abbreviations of nerves are explained in Fig 1 Excitatory action (discharge evoked by

Exp	Nr	SSC	Ax	LHT	B[1]	DR	Med	Uln	SR	Conv
I	1	+ r F	+ F	+	F	+ F	F	+ F	F	5F
	2	−	+	+		F	+ Γ	+ F		5F
	3	−		+			+	+		4
	4	(−)r	− r		F	+		+		3F
	5	(+)	−			+		+		3
	6	F	+ F					+		3F
	7		F	+ F		− F	F	+ F	F	3F
	8		+	+				+		3
	9	F	−		F	+		−		3F
	10	− r F	− F				F	F		2F
	11		− F				+ F			2F
	12	(+ rF		−						1F
	13						+ Γ			1F
II	14	−		+		+		+		4
	15	−				− r F	(+) F	+ r F	F	3F
	16	(−)r F	−			+		+		3F
	17	+ F	− F			F	(−) F	F	F	3F
	18	− r F	− F			−	F	F		3F
	19	− r	−	−						3
	20	+ r I	− F	F		F	F	F	F	2F
	21						−	− r	F	?F
	22		−							2
	23	r	−							2
	24	− r				−				2
	25						(+) F	+ F	F	1F
	26						(+)	+ r F		1F
	27		− r			F	F			1F
	28			−						1
	29	−	−							1
III	30	− F	− F	F	F	− F	F	F	F	3F
	31	− F	− F	F	F	− F	F	F	F	3F
	32	− F	− F	F	F	−	F	F	F	3F
	33	F	− F			−	F			3F
	34			− F		− F	F	− F	F	3F
	35	− F	− F	F	F	F	F	F	F	2F
	36	− F	− F	F						2F
	37	− F	− F							2F
	38	F	F	F	F	− F	F	+ F	F	1F
	39	F	F			− F	F	F	F	1F
	40	F					F	− F		1F

1 Not stimulated in exp II

single volley) is indicated as follows — monosynaptic activation from Group I (+) di or polysynaptic activation from Group I r repetitive activation from Group I F activation from FRA. Monosynaptic excitation from Group I arbitrarily assumed when the latency of the response was less than 1 5 msec as measured relative to the afferent volley recorded at the same segmental level (cf Oscarsson and Uddenberg 1965) Column to extreme right figure number of nerves supplying monosynaptic Group I activation F FRA activation from at least one nerve

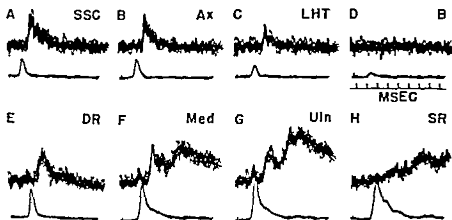


Fig 1 Contribution to RSCT mass discharge from Group I afferents in various nerves. Simultaneous recording from intermediate one third of lateral funiculus (upper traces) and from dorsal funiculus both dissected at the C3 level The muscle component of the suprascapular nerve (SSC) the axillar nerve (Ax) the nerve to the long head of triceps (LHT) the nerve to biceps (B) and the deep radial nerve (DR) were stimulated at maximal Group I strength The median (Med) ulnar (Uln) and superficial radial (SR) nerves were stimulated at 2 5 times threshold strength The initial part of the responses evoked from the two mixed nerves (Med and Uln) can be attributed largely to activity in the RSCT

the mixed nerves (F G) largely represents activity in the RSCT This and similar experiments showed that physiological extensors (SSC, LHT Med) as well as flexors (B DR) contribute Group I excitation to the RSCT The size of the RSCT discharges evoked from the various nerves was roughly proportional to the size of the ingoing Group I volleys

Information about the convergence of monosynaptic excitation to individual RSCT neurones was obtained by recording from single fibres The following discussion is based on observations of 116 units encountered in 15 expts In three of theses 8 ipsilateral nerves could be stimulated and are listed in Table I In the remaining experiments the triceps biceps deep radial and median nerves were usually prepared for stimulation The remarkable degree of convergence found in many units is apparent from Table I Monosynaptic excitation from Group I afferents is indicated by "— There was usually convergence from several nerves and sometimes from as many as five On unit with extensive convergence is illustrated in Fig 2 It was monosynaptically activated from Group I afferents in the suprascapular triceps deep radial and ulnar nerves

Several of the nerves used for stimulation are compound and innervate muscles of diverse function (*cf* Reinhard and Jennings 1935) This obscures the patterns of con

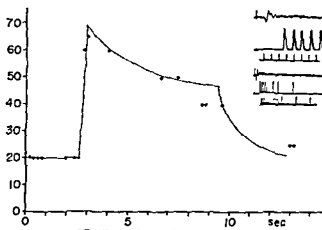


Fig 5 Effect produced in RSCT unit by loading of tendon to muscle supplying FRA but not Group I excitation The unit was activated by stimulation of high threshold muscle afferents in the median nerve and by pull of the tendon to the palmaris longus Inset records show unit response and afferent volley on stimulation of the median nerve at approximately 10 times threshold strength Recording as in Fig 2 The upper and lower pairs of traces were obtained at different speeds Time markers msec The activity (imp/sec) of the unit is plotted against time in sec A steady pull was applied to the tendon during the time indicated by horizontal black bar

typical unit is shown in Fig 3 It was monosynaptically activated from Group I afferents in the triceps deep radial and ulnar nerves (C E H) and polysynaptically activated from the FRA in the axillar triceps deep radial median ulnar and superficial radial nerves There was no obvious correlation between the receptive fields related to Group I afferents and FRA respectively

2 *Natural stimulation of receptors*

a Muscle receptor The effects from receptors in and around muscle were investigated in five experiments The ipsilateral forelimb was denervated except for the deep radial and median nerves which were mounted on stimulating electrodes but left in connection with the periphery The tendons of some of the muscles innervated by these nerves were severed and prepared for pulling or loading (see Methods) RSCT units were identified either by being monosynaptically activated from Group I afferents in the deep radial or median nerve or from Group I afferents in the suprascapular or triceps nerve which were cut and mounted for stimulation

Units activated from Group I afferents but not the FRA in either the deep radial or the median nerve were tested by pulling or loading of the appropriate tendons Activity was usually evoked on pull of one or several tendons but usually not on pull of all the tendons suggesting a certain restriction of the receptive field related to a group of synergistic muscles Most units had a resting activity and the response to maintained pull or to loading was an increased frequency which after a short period of moderate adaptation remained at a steady level (Fig 4) On cessation of loading there was an abrupt decrease in the frequency which returned to the resting level either immediately (Fig 4 B) or after a transient depression (A) A quick stretch of the muscle produced no burst of activity as would be expected if muscle spindle afferents contributed to the

excitation The threshold was only roughly determined Usually a load of 50 to 150 g was necessary for producing an appreciable increase in the resting activity

The RSCT is activated from high threshold Group I afferents in ipsilateral forelimb nerves (Holmqvist *et al* 1963b) The high threshold Group I afferents in hindlimb nerves have been identified as tendon organ afferents in the cat (Bradley and Eccles 1953 Eccles Eccles and Lundberg 1957 Laporte and Bessou 1957) and several other mammalian species (Magni and Oscarsson 1962 Oscarsson Rosén and Uddenberg 1964) The results on receptor stimulation described above indicate that this identification holds true also for the high threshold Group I afferents in forelimb nerves of the cat The properties of the receptors conformed to those of tendon organs slow adaptation high mechanical threshold and no special sensitivity to quick stretch Furthermore the results in a recent investigation (Oscarsson and Rosén 1963) suggest that the low threshold Group I afferents in forelimb nerves originate from muscle spindles

Stretch of muscles supplying FRA but not Group I excitation sometimes produced a short lasting discharge which returned to the resting level after a few seconds However there was usually only moderate adaptation on maintained stretch and cessation of stretch was then followed by an afterdischarge (Fig 5) The receptors responsible for the slowly adapting effects are unkown Group II afferents with flower spray endings might be partly or mainly responsible The afterdischarge was presumably due to a continued activity in the interneurones on the path from the FRA to the tract neurones

RSCT units which seemed to be little affected by stretch of the forearm muscles were sometimes strongly activated by bending of the wrist and toe joints These effects appeared when the joint was rotated towards extreme extension or flexion but before pain receptors were likely to be activated There was usually only moderate adaptation on maintained bending In some cases the foot was dissected for localization of the receptors There was no effect on pressure against the joint capsules or on movement in the individual small joints but usually strong activation on pressure against deep structures in the foot Presumably the receptors were situated in ligaments and other connective tissue

b Cutaneous receptors A volley in the skin nerve, the superficial radial nerve often produced a discharge in the RSCT units (Fig 3 Table I) Stimulation of cutaneous receptors only occasionally evoked activity in these units This activity was produced by pressure pinching and exceptionally also by touch The effects were obtained from vaguely delimited areas on the distal part of the limb (Fig 3) On maintained stimulation there was only moderate adaptation

In many cases stimulation of the skin nerve evoked a distinct or even intense discharge in the RSCT unit while stimulation of cutaneous receptors was ineffective This discrepancy between the effects evoked by a synchronous volley and the asynchronous barrage of impulses on natural stimulation was in some cases observed also in connection with stimulation of high threshold muscle afferents and calls for caution when interpreting receptive fields on the basis of observations made on nerve stimulation

Inhibitory effects were observed in a few units on cutaneous stimulation and on pulling of tendons These effects were weak and of doubtful significance

c Limitation of receptive field Supramaximal stimulation of the contralateral radial nerve was tried with many RSCT units. No discharge was elicited in the vast majority of the units In the remaining units one or two spikes were evoked after a long latency

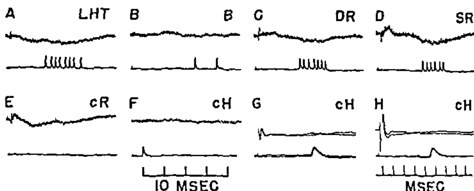


Fig 6 Activity evoked in VSCT unit by stimulation of forelimb nerves The unit response was recorded from fibre in the lateral funiculus at the C3 level The upper traces in A—F show the afferent volleys recorded triphasically from the dorsal funiculus at the sam segmental level A—F stimulation at approximately 10 times threshold strength of the following nerves the ipsilateral nerve to the long head of triceps (LHT) the ipsilateral nerve to biceps (B) the ipsilateral deep radial nerve (DR) the ipsilateral superficial radial nerve (SR) the contra lateral radial nerve (cR) and the contralateral hamstring nerve (cH) The last nerve was also stimulated in records G and H which were obtained at a fast speed and at stimulus strengths producing a submaximal (G) and a maximal (H) Group I volley respectively The upper traces in G and H show the ingoing volley recorded triphasically from a proximal part of the sciatic nerve.

Intense stimulation of superficial and deep receptors in the contralateral forelimb and in the hindlimbs did not influence the RSCT units It is concluded that the receptive fields of the RSCT neurones are ipsilateral like those of other uncrossed tracts (Oscarsson 1964a) and limited to the forelimb

d Supraspinal control In the decerebrate preparation transmission in the FRA paths to motoneurones (Eccles and Lundberg 1959 Holmqvist and Lundberg 1959 1961) primary afferents (Carpenter Engberg Funkenstein and Lundberg 1963) and several ascending tracts (Holmqvist Lundberg and Oscarsson 1960) is tonically depressed This depression is due to a bulbospinal system which descends in the dorsal part of the lateral funiculus In experiments on decerebrate preparations (Oscarsson and Uddenberg 1964) the effects from cutaneous receptors were almost completely lacking in the RSCT units suggesting that the FRA paths to this tract are likewise under inhibitory control The effects evoked from the FRA and described in this paper were tested on unanaesthetized preparations which were decapitated or (2 expts) decerebrated and with the dorsal half of the cord transected in the first cervical segment The latter procedure would interrupt the descending pathways responsible for the bulbar inhibition of FRA paths (Holmqvist *et al* 1960) In the two experiments with intact ventral pathways (*e g* Exp III in Table I) FRA volleys evoked a discharge in the RSCT units more frequently than in the experiments made on spinal preparations (*e g* Exp I and II) The possibility of descending ventral pathways with a facilitatory action on the FRA paths should be considered

3 *Receptive fields of VSCT unit*

Previous investigations had demonstrated that VSCT neurones receive FRA effects from bilateral receptive fields including the hindlimbs and hinder part of the trunk

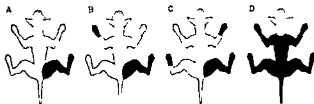


Fig 7 Areas supplying FRA exc tat on (hatched) and inhibition (black) to four VSCT units activated monosynaptically by Group I afferents in the contralateral (right) hamstring nerve

(Oscarsson 1957 Lundberg and Oscarsson 1962) It has now been found that these neurones receive FRA effects also from the forelimbs and front part of the trunk More than 50 VSCT units in 5 expts were identified by their monosynaptic excitation from Group I afferents in the contralateral hamstring nerve One experiment will be de scribed in detail It was made on a decerebrate cat with the dorsal pathways inter rupted in the first cervical segment Twelve VSCT units were encountered Ten of these responded with a train of action potentials on stimulation of all or most of the forelimb nerves tested A typical example is shown in Fig 6 The number of spikes varied from one to more than 10 and the latency (measured from the stimulus artefact to the first spike) was usually between 10 and 20 msec (range 8—50 msec) On repetitive stimulation the VSCT neurones were usually excited but sometimes inhibited as shown by an increase or decrease in the frequency of the resting activity The two units which did not respond to sin le volleys were nevertheless excited or inhibited on repetitive stimulation

The FRA effects from the forelimbs were less readily displayed on natural stimulation of receptors Among the 12 units described above only five were appreciably influenced on pressure or pinching of the skin of the forelimbs The effects from the forelimbs were in general weak they were always weaker than those evoked from the contra lateral hindlimb but sometimes as strong as those from the ipsilateral hindlimb (*cf* Oscarsson 1957) The effects from the forelimbs were excitatory and/or inhibitory in various combinations Some examples of the receptive fields are shown in Fig 7

The FRA effects f om the h ndlimbs have been described before (Oscarsson 1957 1960 Lundberg and Oscarsson 1962) The present observations are ma nly in agreement with the previous ones Thirty five un ts out of 51 were inh b ted from the contralateral hindlimb and e ther excited (28) or not app eciably influenced (7) from the ipsilateral hindlimb (Fig 7 A and B) A larger proportion (13 out of 51) than reported before was inhibited not only from the contralateral but also from the ipsilateral hindlimb (Fig 7 C and D) and one unit was ex c ted from the contralateral and inhib ted from the ips lateral side The latter combination of effects has not been described p ev ously Of the remaining 2 units one was excited from both h ndl mbs and the other not influenced from either h ndlimb

The effects from the forelimbs did not depend on pathways throu h the brain stem *Similar effects were observed in the spinal preparations However* it is possible that descending ventral pathways from the brain stem facilitate the FRA effects (*cf* above) Natural stimulation of receptors in the forelimbs was observed to influence the VSCT units more often in the 2 expts with these pathways intact than in the three experiments made on high spinal animals

The effects evoked in VSCT neurones by stimulation of cutaneous receptors in the contralateral hindlimb were stronger than the effects evoked in RSCT neurones by

stimulation in the ipsilateral forelimb The significance of this finding is obscure The FRA paths to the RSCT neurones might depend more on facilitation from supraspinal centres than the corresponding paths to the VSCT neurones

Discussion

The conclusion that the RSCT is a functional equivalent of the VSCT (Oscarsson 1964b Oscarsson and Uddenberg 1964 1965) is supported by the present findings Both tracts are monosynaptically activated by high threshold Group I afferents which have been identified as tendon organ afferents The convergence of Group I excitation to individual neurones is as extensive in the RSCT as in the VSCT The patterns of convergence have been studied less in the RSCT than in the VSCT but the available evidence indicates that these patterns are similar in the two tract There is often excitation from one synergic muscle group at each of several joints and sometimes from one group at each of the main limb joints Furthermore in both tracts co activation from antagonist muscle groups presumably does not occur though some observations on the RSCT might indicate exceptions to this rule The tract neurones are presumably activated from muscles that contract together in the execution of a certain movement or the maintenance of a certain posture (Oscarsson 1960 Eccles *et al* 1961) The RSCT and VSCT would carry information concerning stages of movement or position of the whole limb rather than information about change of tension in individual muscles

Both tracts receive FRA actions from large receptive fields In the RSCT the effects were relatively weak and predominantly excitatory whereas they were strong and predominantly inhibitory in the VSCT The difference in intensity of the effects in the two tracts might be related to a differential need for facilitation from higher centres The fact that the FRA effects are predominantly excitatory in the RSCT and predominantly inhibitory in the VSCT might be of little general significance Some VSCT units receive predominantly excitation in the cat (Oscarsson 1957 Lundberg and Oscarsson 1962) and excitation is the dominant effect in the VSCT of the dog (Oscarsson *et al* 1964)

The same problems arise in assessing the significance of the information forwarded by the RSCT because of its FRA connections as arise in connection with other ascending pathways influenced by the FRA It has been suggested that these tracts carry information concerning flexor reflex patterns for example by reflecting the degree of motoneuronal activation from interneurones on the paths from the FRA This would require that the interneurones transmitting effects from the FRA to motoneurones and to ascending tracts are influenced in a similar way not only from the periphery but also from higher centres (Lundberg 1959 Holmqvist *et al* 1960 Lundberg Norsell and Voorhoeve 1963) Experiments on the supraspinal control exerted by the pyramidal tract (Magni and Oscarsson 1961 Lundberg *et al* 1963) and by a bulbospinal system (Holmqvist *et al* 1960) demonstrate that this is indeed the case in some instances

With the RSCT there are obvious difficulties with the above hypothesis because of the weak effects from the skin and relatively strong effects on moderate stretch of muscle However these features might be due to the experimental situation in the intact animal with normal supraspinal control the FRA effects may be different With the VSCT this hypothesis might explain some observations made during the present investigation The VSCT neurones were shown to be influenced from the FRA not only in the hindlimb nerves but also in the forelimb nerves though the effects from the latter

were weak and variable The interneurones on the paths from the FRA in hindlimb nerves to lumbo-sacral motoneurones are influenced by the pyramidal tract and it has been suggested that the pyramidal effects on the motoneurones are exerted through facilitation and inhibition of segmental reflex arcs (Lloyd 1941 Lundberg Norsell and Voorhoeve 1962 Lundberg and Voorhoeve 1962) It is not unlikely that these interneurones are influenced also from the FRA in the forelimbs and at least in part responsible for the effects that these afferents exert on lumbo-sacral motoneurones (*cf* Lloyd 1942 Lloyd and McIntyre 1948) If the VSCT forwards information concerning the transmittability in these interneurones the effects from the forelimbs would be readily explained

This paper concludes for the time being our series of articles on the RSCT It has been shown that this tract is a forelimb equivalent of the VSCT though a separate anatomical entity The RSCT and VSCT might have developed independently from different origins by parallel evolution of a similar pattern of organization (Oscarsson and Uddenberg 1965) The information forwarded by the two tracts is related to two systems of primary afferents the tendon organ afferents and the FRA Presumably the integrated information from these two systems is of special value for the cerebellum (Oscarsson 1960) and cannot be replaced by information through separate information channels for tendon organ and flexor reflex afferents Such channels are available in the DSCT and presumably also in the cuneocerebellar tract (Lundberg and Oscarsson 1960 Holmqvist Oscarsson and Rosén 1963a)

Many problems concerning the RSCT remain to be solved The tract has not been identified with anatomical methods though such work is in progress (G Grant personal communication) Localization of the cell bodies and intracellular recording from them is desirable and would permit a detailed study of the synaptic properties and the patterns of convergence The organization of the VSCT has been investigated in several mammalian species (Magni and Oscarsson 1962 Oscarsson *et al* 1964) but the RSCT is known only from experiments on the cat Comparative studies might provide clues to the functional significance of the tract It is of interest that recent observations on the duck suggest that the RSCT and VSCT might exist in the bird and have a similar organization as in mammals (Oscarsson Rosén and Uddenberg 1963)

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From the Institute for experimental Medicine University Copenhagen Denmark

The in Vivo Effect of Angiotensin and Noradrenaline on the Proximal Tubular Reabsorption of Salt in Mammalian Kidneys

By

PAUL P LEYSSAC

Received 6 Oktober 1964

Abstract

Leyssac P P *The in vivo effect of angiotensin and noradrenaline on the proximal tubular reabsorption of salt in mammalian kidneys* Acta physiol scand 1965 *64* 167—175 — The purpose of the investigation presented here was to study the immediate effect of angiotensin and of noradrenaline on the rate of proximal tubular reabsorption of filtrate in mammalian kidneys by a direct method By registration of the occlusion time which is the time interval between interruption of the renal circulation and the moment at which the majority of the proximal tubules have completed reabsorption of the luminal fluid it is demonstrated that angiotensin causes a depression of the tubular reabsorption rate which is maximal when about 0 7 × 10 g is acting per g of kidney In contrast noradrenaline was without any immediate effect on the proximal reabsorption rate suggesting that effects observed in clearance studies are indirect and mediated by a rapid intrarenal mechanism possibly involving the angiotensin system.

Clearance studies on the effects of noradrenaline (*e g* Jacobsen Hammersten and Heller 1951 Smythe Nickel and Bradley 1952 Berne *et al* 1952 Pullman and McClure 1954) and of angiotension (*e g* Book and Krecke 1958 Gross and Turrian 1959 Finnerty 1962) on salt excretion have shown that the renal response to these pressor substances is qualitatively identical angiotensin being about 10 times more potent The typical acute effect in dogs and human subjects a decrease in urine flow sodium excretion and renal blood flow with a more moderate reduction in the glomerular filtration rate has generally been attributed solely to the vascular action of the pressor substances Since no correction for dead space error have been carried out in these clearance studies in which a considerable reduction in urine flow occurred as a response to the administration of the pressor substances a gross overestimation of the early reductions in glomerular filtration rate is introduced unless the initial one or two 10-min clearance periods are critically evaluated However even with due respect to such likely gross errors it appears that at least during the initial 20—30 min of the experiments before late secondary compensatory mechanism (*e g* aldosterone) may be

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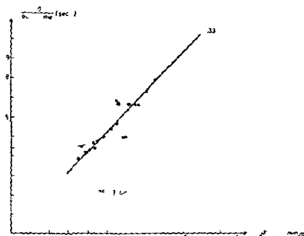


Fig 1 Reciprocal occlusion times (u) at spontaneously different rates of glomerular filtration (v) in control rats

in the kidneys and in the 10 samples of the injected dose were counted in a well scintillation counter connected to an impulse height analyser

Analyses of inulin were performed by the method of Bojesen (1952) modified for micro-analysis

Results and calculations

Fig 1 shows the pooled control data of the reciprocal occlusion time (u) plotted against the means of 2—4 determinations of inulin clearance (v) measured immediately before the registration of occlusion time Included in the figure are also data published previously (Leyssac 1963) By the method of linear regression analysis disregarding points with (v) > 1 6 ml/min/g KW because they are few and scattered the relationship between reciprocal occlusion time and the clearance of inulin was found to be represented by a straight line which did not differ significantly from a line described by (u) = 5 33 (v)

It appears that the spontaneous variation in clearance of inulin ranges between about 0 7 and 1 7 ml/min/g KW with an accumulation of observations around 1 2 ml/min/g KW Below this range of spontaneous variation a horizontal broken line has been drawn corresponding to the maximum occlusion time observed following the administration of 25 ng of synthetic angiotensin (Fig 2) Although not included in the statistical analysis a few observations at very high inulin clearances suggest a deviation from a direct proportionality between (u) and (v) or a systematic error in evaluation of one or both of these parameters at these high rates of reabsorption

The immediate effect of three doses of synthetic angiotensin on the reciprocal occlusion time at spontaneously different clearances of inulin is demonstrated in the Fig 2 to 4 It appears that an effect is clearly demonstrated at all three dose levels increasing with the dose administered until a maximum of occlusion time (about 23 sec) is reached by the injection of 25 ng of angiotensin This maximum equals the average maximal

operative the absolute decrease in the rate of glomerular filtration of sodium by far exceeded the fall in sodium excretion indicating a decreased rate of overall reabsorption Since it has been demonstrated (Leyssac 1963) that the rate of proximal tubular reabsorption of salt (which normally accounts for about 75 per cent of the total reabsorption) is independent of the filtered load eo ipso (i e that the proximal reabsorption is a transport process always operating at its T max the level of which is physiologically variable) the change in the proximal tubular sodium T max must have been due either to a direct action of the pressor substances or due to an indirect action mediated by a rapid intrarenal regulatory mechanism

It was previously observed (Leyssac 1964) that angiotensin has an immediate and direct depressing effect on the proximal reabsorption rate (T max) by which this rate may be reduced to a certain minimum level equal to the lowest value in the range of physiological variation The purpose of the present investigation was to determine the dose of angiotensin necessary to depress the sodium T max to its minimum and to investigate whether a similar direct tubular effect of noradrenaline could be demonstrated The results have indicated that about 0 7 × 10 g (0 7 ng) of angiotensin acting per g of kidney is capable of eliciting an almost maximum depression of the proximal tubular reabsorption rate in rat kidneys of about 0 8 g No immediate tubular effect of noradrenaline could be demonstrated suggesting that its effect on the renal tubular handling of sodium is indirect and mediated by an intrarenal regulatory mechanism most likely involving the renin angiotensin system

Methods

Male albino rats weighing about 250 g were anesthetized and prepared for determination of occlusion time and measurement of inulin clearances from the exposed left kidney as described in details elsewhere (Leyssac 1963 1964) For testing the tubular effect of the two pressor substances either synthetic angiotensin (Val 5-angiotensin amide CIBA) or noradrenaline was given as a single intravenous injection immediately after the last collecting period for clearance determinations At the very moment when the pressor substance reached the kidney that is as the kidney began to pale the aorta was totally clamped and the occlusion time was noted Angiotensin was administered in doses either of 25 ng 7 15 ng or 5 ng in 0 0?0 ml of saline Noradrenaline was given in doses of 400 to 600 ng in 0 0?0 ml of saline

In some experiments the occlusion time was registered immediately after the last urine collecting period without administration of pressor substance (controls) The tubules were then allowed to refill by opening the clamp angiotensin or noradrenaline was given after an interval of about 2 min and the occlusion time was registered again In other experiments after the administration of noradrenaline or angiotensin and the determination of occlusion time the tubules were allowed to refill and 2 to 3 min after the first measurement of the occlusion time the aorta was clamped again and the occlusion time noted without giving any more pressor substance These latter determinations of occlusion time are also included in the control determinations When more than one injection of pressor substance was given to the same animal subsequent injections were given after an interval of at least 15 min in which time urine was collected for repeated clearance determinations

In another series of experiments a single intravenous injection of I labelled albumin (0 15 mg 0 035 μC in 0 0?0 ml of saline was given either alone or with 10 or 25 ng of angiotensin or with 500 ng of noradrenaline Instead of registration of occlusion time the left kidney was removed 9 sec after start of the injection (i at the moment when the kidney starts to pale equal to about 3 –5 sec after completion of the injection) and immediately frozen (within 1 sec) in isopentane cooled to about 160 with liquid nitrogen Each of the frozen kidneys were transferred to test tubes and were equilibrated at room temperature with distilled water added to a total volume 1 1 ml Of the same solution as administered to the animals 0 0?0 ml were injected with the same syringe into 10 test tubes containing 1 0 ml of water I activity

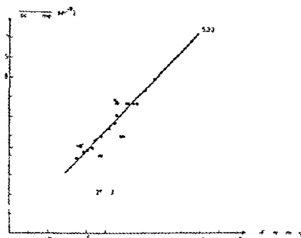


Fig 1 Reciprocal occlusion times (u) at spontaneously different rates of glomerular filtration (v) in control rats

in the kidneys and in the 10 samples of the injected dose were counted in a well scintillation counter connected to an impulse height analyser

Analyses of inulin were performed by the method of Bojesen (1952) modified for microanalysis

Results and calculations

Fig 1 shows the pooled control data of the reciprocal occlusion time (u) plotted against the means of 2—4 determinations of inulin clearance (v) measured immediately before the registration of occlusion time Included in the figure are also data published previously (Leyssac 1963) By the method of linear regression analysis disregarding points with (v) > 1 6 ml/min/g KW because they are few and scattered the relationship between reciprocal occlusion time and the clearance of inulin was found to be represented by a straight line which did not differ significantly from a line described by (u) = 5 33 (v)

It appears that the spontaneous variation in clearance of inulin ranges between about 0 7 and 1 7 ml min g KW with an accumulation of observations around 1.2 ml/min g KW Below this range of spontaneous variation a horizontal broken line has been drawn corresponding to the maximum occlusion time observed following the administration of 25 ng of synthetic angiotensin (Fig 2) Although not included in the statistical analysis a few observations at very high inulin clearances suggest a deviation from a direct proportionality between (u and v) or a systematic error in evaluation of one or both of these parameters at these high rates of reabsorption

The immediate effect of three doses of synthetic angiotensin on the reciprocal occlusion time at spontaneously different clearances of inulin is demonstrated in the Figs 2 to 4 It appears that an effect is clearly demonstrated at all three dose levels increasing with the dose administered until a maximum of occlusion time (about 23 sec) is reached by the injection of 25 ng of angiotensin This maximum equals the average maximal

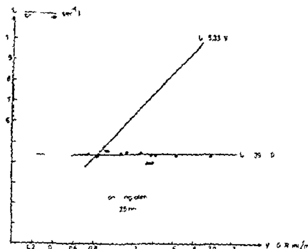


Fig 2 Reciprocal occlusion times after injections of 25 × 10 g of angiotensin at spontaneously different rates of glomerular filtration measured immediately before the administration of angiotensin

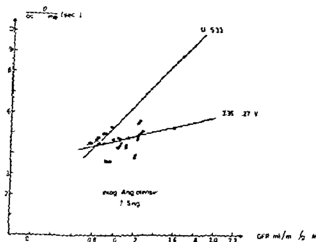


Fig 3 Reciprocal occlusion times after injections of 7 15 × 10 g of angiotensin at spontaneously different rates of glomerular filtration measured immediately before the administration of angiotensin

occlusion time observed spontaneously (Fig 1) which corresponds to an inulin clearance of about 0 85 ml/min/g KW

The indication of a maximal tubular effect caused by angiotensin suggests a dose response relationship with horizontal asymptote A quantitative description of the observed effect derived from a relationship of this form, according to which the regression lines given in Fig 2—4 are calculated will be published elsewhere It does however appear directly from the figures that the amount of exogenous angiotensin

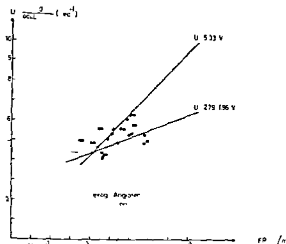


Fig 4 Reciprocal occlusion times after injections of 5 × 10 g of angiotensin at spontaneously different rates of glomerular filtration measured immediately before the administration of angiotensin.

necessary to reduce the inulin clearance of a rat kidney from 1 60 to 0 85 ml/min/g KW is about 20 × 10 g

By injection of ¹³¹I labelled albumin with 10 ng of synthetic angiotensin in 0 050 ml of saline removal and snap-freezing of the left kidney 9 sec after start of the injection and subsequent registration of the radioactivity in the kidneys and in 10 samples of 0 050 ml of the injected solution, the activity in the kidneys in per cent of the injected amount of activity was found to be

$$\frac{(419 \pm 30 \text{ c/m})}{(13\ 450 \pm 250 \text{ c/m})} \times 100 = 3\ \%$$

This fraction of the injected dose was independent of the concentration of pressor substances since identical values (2—4 per cent) was found no matter whether albumin was given alone or with angiotensin in doses from 10 to 25 ng or with 500 ng of nor adrenaline Assuming that also 3 per cent only of the injected amount of angiotensin was present in the kidney at the time of registration of its effect and that the spontaneous variation in the rate of proximal reabsorption is determined by the concentration of endogenous angiotensin the data indicate that the difference in the amount of endogenous angiotensin in a rat kidney which may be responsible for the physiological interval of variation equals about 0 6 ng (0 3—0 9) or about 0 7 ng (0 35—1 0 ng)per g kidney weight since the kidney weight of 250—300 g rats was found to be 0 87 ± 0 08 g Since the cortex constitutes the major part of the rat kidney (about 80 %) and has a blood flow per g of tissue at least not less than the medulla and since the tubular cells make up some 60 per cent of the cortical volume it may also be assumed that some 1 2 ng of angiotensin (= 1.2 × 10⁻¹² moles or about 7 10¹¹ molecules) would be required per g of tubular cells to cause a maximal depression (by angiotensin) of the glomerular filtration rate Assuming the cells to be cubic with a thickness of 6—7 microns about 200 molecules per cell would account for a depression of the filtration rate from the upper to the lower limit of the spontaneous range of variation

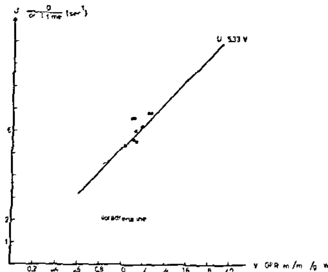


Fig 5 Reciprocal occlusion times after injections of 4—6 × 10 g of noradrenaline at spontaneously different rates of glomerular filtration measured immediately before the administration of noradrenaline

Since pilot experiments with noradrenaline in doses of 100 ng failed to demonstrate any effect on the occlusion time the present experiments were carried out with pressor doses of noradrenaline (400—600 ng) exceeding those of 25 ng of angiotensin, which did provoke an almost maximal effect With both pressor substances the same fraction of the injected dose (2—4 per cent) may be assumed to have been present in the kidney at the instant of registration of the effect It appears from Fig 5 that even such heavy doses are without any effect at all on the occlusion time The observed relationship between reciprocal occlusion time following noradrenaline and the (preceding) clearance of inulin does not differ from the regression line (u) = 5 33 (v) observed when no drugs were administered

Discussion

A. *The relationship between the rate of proximal reabsorption and the glomerular filtration rate*

In continuation of previous results (Leyssac 1963) the pooled control data have confirmed a linear relationship between the reciprocal occlusion time representing the rate of proximal reabsorption and the inulin clearance within the range of physiological variation However at rates of filtration well below this range of spontaneous variation a deviation from the linear relationship was observed in partially clamped kidneys (Leyssac 1964) It was demonstrated in acute experiments that below filtration rates of 0 8—0 7 ml/min/g KW occlusion time did not exceed a maximum value significantly different from that observed following the injection of a high dose of synthetic angiotensin no matter how far the filtration rate was reduced Thus it was suggested that a similar maximum inhibition on the proximal salt T max was caused by endogenous angiotensin as that elicited by exogenous angiotensin In Fig 1 this maximum inhibition is indicated by the interrupted line at filtration rates below 0 8 ml/min/g KW

B *The tubular response to angiotensin*

Evidence was presented previously (Leyssac 1964) that the injection of a high dose of angiotensin had not to any significant degree affected the luminal diameter of the proximal tubules at the moment of clamping the aorta (i e 9 sec from start of the injection) It was further stated that the instantaneous renal vasoconstriction induced by the substance on principle being equal to the interruption of the renal circulation by clamping the aorta or the renal artery did not itself affect the proximal rate of reabsorption as evaluated from the occlusion time This statement is quite established by the present demonstration that doses of noradrenaline with a reno-vasoconstrictive effect exceeding that of doses of angiotensin causing maximal inhibition of the reabsorption rate does not influence the rate of reabsorption The dose-dependent depression of this rate as demonstrated following injections of angiotensin must therefore be due to a direct tubular effect in accordance with previous conclusions

By simultaneous injection of labelled albumin evidence is presented that only a minor fraction (about 3 per cent) of an injected dose has actually reached the kidney at the moment of clamping the aorta Since the vasoconstriction (and hence the paling is almost instantaneous and since the aorta is clamped at the very moment when the kidney starts to pale it is unlikely that either labelled albumin or exogenous angiotensin has left the kidney by the venous route at the instant of freezing Disregarding a possible overestimation in assuming that all of the molecules present in the kidney has actually reached the effector sites at the moment of registration of their effect the results indicate that a maximum of change in the proximal salt T max may be elicited by a change in the amount of angiotensin of about 0 6 ng per rat kidney Since the kidney is highly sensitive to a change of this order of magnitude one would expect that the endogenous amount of free angiotensin in the kidney is of a similar order of magnitude The present data therefore suggest that an order of magnitude of some hundred molecules of angiotensin per cell is sufficient to elicit a maximum physiological depression of the rate of proximal tubular reabsorption This reasonably low value would seem to support the previous conclusion (Leyssac 1964) that angiotensin is a physiological regulator of the proximal salt T max

C *Interpretation of results obtained with angiotensin in clearance studies*

Different or even directly opposing tubular effects of angiotensin has been concluded from indirect evidence obtained in clearance studies In accordance with the ideas of Smith (1951) the typical response (i e reduced excretion rate of sodium to renin angiotensin or noradrenaline in dogs and normal human subjects was generally ascribed to the vasoconstrictive action of the pressor substances or based upon it seems most insignificant evidence to an increased sodium reabsorption (Biron *et al* 1962 Laragh *et al.* 1963) In contrast to the typical response to angiotensin or noradrenaline a marked increase in sodium excretion rate in response to these substances was demonstrated in rabbits and rats (Pickering and Prinzmetal 1940 Huges Jones *et al* 194 Eversole Giere and Rock 195 Peters 1963 in hypertensive humans Peart and Brown 1961 Biron *et al* 196 and in patients with cirrhosis and ascites Laragh *et al* 1963) Renal plasma flow was clearly decreased whereas changes in glomerular filtration rate were less significant The response in these species of animals and in patients with hypertension or secondary aldosteronism was ascribed to an inhibition of tubular sodium reabsorption Taking into account the T max character of the reabsorp-

tion and the very small amount of sodium excreted per min relative to the rate of glomerular filtration of sodium these conclusions would seem unjustified irrespective of inevitable errors in the clearance measurement Even disregarding such errors the clearance data presented (e g Biron et al 1962) show that the absolute amount of sodium reabsorbed per unit time was reduced following the administration of angiotensin not only in hypertensive patients but also in normal subjects Thus it is more likely that angiotensin has exerted a depressing effect on the rate of reabsorption (proximal T max of salt) in both groups According to this interpretation the different effects on the rate of sodium excretion observed in the two groups of patients and between different species of animals differences of no more than a few hundred μeq Na per min corresponding to a few ml of glomerular filtrate would be explained by the combination of slightly different arteriolar responses to angiotensin in the two groups with a similar (maximal) depressing effect on the proximal reabsorption rate i e a condition of disturbed glomerulo-tubular balance conditioned by the combined vascular and tubular effect of angiotensin The difference in vascular response should only account for a difference in filtration pressure sufficient to change filtration rate a few ml per min all other factors being equal A decreased pressor response to angiotensin was demonstrated in experimental secondary aldosteronism (Davis Carpenter and Ayers 1967) and a change in the excretion rate of sodium in response to angiotensin was also demonstrated in patients with secondary aldosteronism and in hypertensives in whom the excretion of aldosterone may often be somewhat increased (Laragh, Cannon and Ames 1963 Genest 1963) In hypertensive patients other factors than aldosterone may possibly further change the vascular response to angiotensin

In this context it should be emphasized that the filtration rate was reduced in normal subjects during infusions of angiotensin from control values of about 120 ml/min to about 80 ml/min In hypertensive patients control values of about 80 ml/min (still within the normal range) only changed to about 75 ml/min during the infusion (Biron et al 1962) A similar reduction from 120 to 80 ml/min in normal subjects was reported by Finnerty (1962) These observations agree with the present direct evidence of a maximum effect of angiotensin on the proximal salt T max which reduces the T max to the minimum value observed spontaneously

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From the Institute for experimental Medicine University Copenhagen Denmark

The Luminal Occlusion Time of Proximal Tubules in Kidneys of Young Rats

By

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Abstract

Leyssac P P *The luminal occlusion time of proximal tubules in kidneys of young rats* Acta physiol scand 1965 64 176—181 — The purpose of this investigation was to test the validity of the luminal occlusion time as a method for estimation of the rate of proximal reabsorption The occlusion time denotes the time required from interruption of the renal circulation until the proximal tubules have completed reabsorption of the luminal fluid The results demonstrate that the close correspondance between the rate of reabsorption as evaluated from the occlusion time and the immediately preceding clearance of inulin holds also in experiments with young rats of a body weight of 100 g in which the volume of luminal fluid per unit tubular length is far less than in kidneys of adult rats

Measurements of the luminal occlusion time defined as the time required from interruption of the renal circulation and filtration until the proximal tubules have completed reabsorption of the luminal filtrate has been used as a relative estimate of the volume of proximal fluid reabsorbed per unit time and unit tubular length i e of the rate of proximal reabsorption (Leyssac 1963) Provided that the volume of fluid per unit tubular length of the proximal tubules (i e the internal diameter) is kept reasonably constant from one experiment to the other by using animals of the same body weight and kidney weight in which the number and dimensions of the proximal convoluted segments should be equal a difference in the occlusion time should directly indicate a difference in the rate of transcellular transport of salt or salt T max

The validity of the occlusion time method as a measure of the proximal reabsorption rate was based mainly on the demonstration by Hanssen (1960) that during the luminal occlusion filtered ferrocyanide was neither displaced to any significant degree towards the distal part of the nephron nor in direction of the glomerulus Supporting evidence was given by the close accordance between the measured occlusion time and the expected time necessary to empty the proximal lumina as calculated from the rate of glomerular filtration and the dimensions of the proximal tubules (Leyssac 1963)

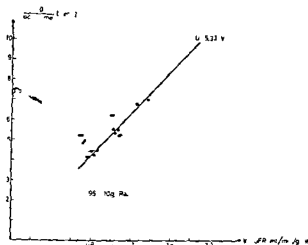


Fig 1 Reciprocal occlusion time at spontaneously different rates of glomerular filtration in 100 g rats The regression line u = 5 33 v was calculated from similar observations in 250—300 g rats

As an attempt to verify the validity of the method the purpose of the present investigation was to see whether the close correspondance between the rate of reabsorption as evaluated from the measured occlusion time and the immediately preceding clearance of inulin holds also in cases in which the volume of fluid per unit tubular length was different from that of previous experiments

After the earliest post embryonic stages the growth of the kidney depends solely on the growth of the tubules which predominantly is due to an increase in the size of the proximal segment as shown by Sperber (1944) in maceration specimens Measurements in vivo by Steinhausen *et al* (1963) of the internal diameters of proximal tubules have further shown that the diameters of adult rats are larger than those of young rats Therefore in the present investigation the relationship between reciprocal occlusion time and rates of glomerular filtration was studied in the range of spontaneous variations in young rats and the internal diameters and cellular areas of the proximal tubules were measured It is shown that the relationship between occlusion time and rate of filtration per g kidney weight (KW) does not differ significantly from that observed previously in adult animals (Leyssac 1963 1965) in agreement with expectancy from the measured diameters and cellular areas

Methods

Male albino rats weighing 90—100 g were anesthetized and prepared for determination of occlusion time and measurement of inulin clearances from the exposed left kidney as described previously (Leyssac 1963 1964)

In another series of experiments the left kidney was quickly removed instead of clamping the aorta and frozen within on sec from removal at — 160 C in isopentane cooled with liquid nitrogen The frozen kidneys were transferred to absolute ethanol at — 20 C for freeze substitution and thereafter embedded in paraffin sectioned (3 μ thickness) and stained with periodic acid Schiff (MacManus Histological sections for measurement of internal diameters

TABLE I. Shows the ± stand. deviations of the mean of kidney weights and internal diameters measured in the two groups of rats. Numbers in brackets give the range of values. The number above the mean value gives the number of kidneys examined.

Body weight	Kidney weight	Luminal Diameter	Calculated Luminal area
95—110 g	26 0.429±0.096 g	6 19.0± 3.2 μ (17.3—22.2)	284 μ
250—200 g	100 0.874±0.079 g	6 27.1± 1.7 μ (25.4—28.8)	573 μ^2

were prepared from two groups of rats with body weights of 90—110 g and 250—300 g respectively. The internal diameters and cellular areas of the proximal tubules were measured by means of a micrometer ocular and planimetry respectively in 50 circular (i.e. transversely cut) proximal tubules from the superficial cortex of each kidney.

Results

In the figure the reciprocal occlusion time (u) is plotted against the means of 2—4 determinations of inulin clearance (v) measured immediately before the registration of occlusion time. In the figure is also given the regression line (u) = 5.33 (v) calculated from data obtained in experiments on adult rats (Leyssac 1965). It is seen that the observations registered in the small rats are distributed along this regression line which passes through (0,0).

The line through (0,0) best fitting the present observations disregarding observations with (v) > 1.60 ml/min/g KW has a slope of $\bar{u}/\bar{v}$ = 5.32, i.e. its equation (y = b(v)) is y = 5.32 (v) with a variance of y (s_y^2) = 0.965 and a variance of b (s_b^2) = 0.016 and 39 degrees of freedom. This line does not differ significantly from the line y = 5.33 (v) found for adult animals in the main interval of physiological variation.[1]

Since the number of nephrons does not increase with growth after the early post natal period (Sperber 1944), a certain glomerular filtration rate (GFR) per g of kidney weight (KW) represents in small rats (with an average kidney weight of 0.43 g) a filtration rate per nephron about half of that of adult kidneys (of a kidney weight of 0.87 g).

From the data given in the table it appears that the transsectioned proximal luminal *area in kidneys of young rats was about half of that in adult rat kidneys.* Consequently the volume of tubular fluid per unit tubular length in the small rats was also half of the luminal volume in adult rats. Since furthermore the kidney weight in the small animals was also half of that in adult rats, it follows that the volume of fluid per unit tubular length is approximately proportional to the kidney weight.

The ratio cellular area to luminal area, which was found to be on an average *1.18 in* small rats and 1.05 in adult rats did not differ significantly (p > 0.05).

[1] The statistical analysis was carried out by Inger Harder Hansen, to whom the author is deeply indepted.

Discussion

By the present technique of snap-freezing and histological procedure which was adopted from the investigations of Hanssen (1960) it seems possible to obtain a histological picture of the renal cortex which corresponds with some reservations rather closely to the conditions in vivo. From the internal diameters of transsectioned proximal tubules it is calculated that the luminal area in 100 g rats is about half of the luminal area of proximal tubules in rats weighing 250—300 g in close agreement with the observations in vivo reported by Steinhausen *et al* (1963). Also the present absolute values of proximal internal diameters in the two groups of rats (19 μ and 27 μ respectively) agree reasonably well with those measured by Steinhausen *et al* as they found that the proximal internal diameters in vivo were 16—17 μ in 80 g rats and 23 μ in 300 rats. Thus the luminal diameters measured in the histological sections of both groups of rats were slightly larger than those observed in vivo. The small discrepancy may in part be due to differences in the experimental conditions (degree of hydration etc.) but it can in the main be accounted for by the fact that the brush border was included in the present measurements because it is less well-defined in the histological sections whereas this was not the case in the in vivo measurements.

The real luminal volume should probably be calculated from a value between these two extremes.

If the total luminal volume of the proximal convoluted segment is denoted by V, the volume of fluid reabsorbed per sec in this part of the proximal tubule during the luminal occlusion by V and the occlusion time by occl t, we have that

$$V = V \times \text{occl t.} \tag{1 a}$$

or

$$\frac{1}{\text{occl t}} = \frac{V}{V} \tag{1 b}$$

If we denote the fraction of filtrate normally reabsorbed by the convoluted proximal segment by F and make the assumption that the reabsorption continues in this segment at an unaltered rate after interruption of the renal circulation and filtration we also have that

$$\frac{1}{\text{occl t}} = \frac{F}{V} \times \text{GFR} \tag{2 a}$$

or

$$\frac{1}{\text{occl t}} = \frac{F}{\left(\frac{V}{\text{KW}}\right)} \cdot \frac{\text{GFR}}{\text{KW}} \tag{2 b}$$

Thus the relationship between reciprocal occlusion time and GFR per g KW should remain unchanged with equal values of F and $\frac{V}{\text{KW}}$. The present data indicate that this relationship does not differ significantly in young and adult rats (equal slope = equal $\frac{F}{\left(\frac{V}{\text{KW}}\right)}$). If it may be assumed that the fractional reabsorption of filtrate in the proximal convoluted segment (F) is approximately the same in young and adult rats which seems reasonable $\frac{V}{\text{KW}}$ should be equal in the two groups of rats investigated if the

general assumption of unaltered rate of reabsorption during the luminal occlusion was valid Taking into account that the proximal tubules constitute the major fraction of the kidney weight, $\frac{V}{KW}$ actually seems to be equal in young and adult rats since the ratio luminal area to cellular area was found to be approximately equal in the two groups. The present data therefore support the previous conclusion that the proximal re absorption continues at an unaltered rate after cessation of filtration (Leyssac 1963)

The rather closely coinciding completion of the luminal occlusion of all the tubules in a field of vision indicates that all visible parts of the proximal convolutions equal to the first two thirds of this segment complete reabsorption almost simultaneously (within 4—5 sec) Since it was demonstrated by Hanssen (1960) that the proximal fluid is not to any significant degree displaced either distally or in direction of the glomerulus following interruption of the circulation, a well-defined end point of occlusion of the whole visible fractions of these segments indicates a very uniform reabsorption rat throughout this part of the tubules This conclusion is in disagreement with some inter pretations of recent micropuncture data. Lassiter Gottschalk and Mylle (1961) sug gested a linear relationship between proximal tubular fluid to plasma ratios of inulin and the distance of collection from the glomerulus from results obtained in non diuretic rats which would indicate a rapidly decreasing rate of reabsorption along the convoluted segment Because of a wide scatter of observations a linearity was however not established In later reports by Lassiter Mylle and Gottschalk (1964) and by Giebisch, Klose and Windhager (1964) on saline diuretic rats the observed proximal inulin ratios were said to be consistent with a model presented by Kelman (1962) In this model the proximal tubule is considered to be analogous to a catalytic flow reactor in which reaction velocity or rate of fluid reabsorption per unit length of the proximal tubule is proportional to the rate of fluid flow through the tubule the flow of which therefore decreases exponentially with the distance from the glomerulus This model thus is in general agreement with the ideas of Smith (1951) This model requires that a semilogarithmic plot of the inulin ratios against distance from the glomerulus should yield a straight line passing through (1 0) This was however apparently not the case In both reports the data may be fitted to a straight line, but this line clearly intercepts with the ordinate (TF P inulin) at a value significantly lower than unity The reported data, therefore are obviously incompatible with an exponentially decreasing reabsorption rate along the proximal convoluted segment but seem to fit very closely the theoretical curve calculated from the assumption of equal reabsorption rates along this segment of the tubules Also the linear relationship between percentile proximal fluid reabsorption and the distance from the glomerulus calculated from the data of Walker *et al* (1941) and from the observations by Wind hager and Giebisch (1961 Fig 3) and by Giebisch, Klose and Winhager (1964 Fig 1) indicate a uniform reabsorption rate along the proximal convolutions Thus micro-puncture data as well as the well-defined end point of the luminal occlusion further support the concept that the rate of proximal reabsorption is independent of the "filtered load" i e that it has the character of a T max process as suggested by Bojsen (1954) and indicated by previous results from this laboratory (Leyssac 1963)

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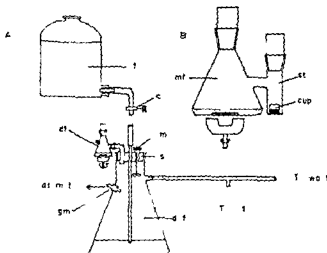


Fig 1 A Experimental set up for administering a constant concentration of CO to spontaneously breathing rats A given amount of Na CO (200 μc) was pipetted into a 50 ml glass flask (df) equipped with 2 side arms and a glass stopper The flask was connected to the interior of a 12 litre Buchner flask (def) via a short glass tube fitted into a rubber stopper in the 12 litre flask In order to obtain gaseous CO_2 in the delivery flask (def) acid was injected through a rubber stopper in a side tube in the diffusion flask (df) and the content of the latter flask was stirred magnetically The attainment of a diffusion equilibrium (controlled with continuous measurement of the CO activity in the flask by means of a thin window GM tube) was aided by means of a fan inside the delivery flask (fm) After completed diffusion a clamp was opened on a rubber tubing connecting the delivery flask (def) with a reservoir flask (rf) containing glycerol The radioactive gas mixture which was displaced by the glycerol flowed through a rubber tubing from the delivery flask (def) to a fan The rats were connected to the gas mixture by joining directly the tracheal cannula to a T tube in the outlet rubber tubing *B* Diffusion unit for absorption of the CO in the tissue into Hyamine 10X The frozen and crushed tissue was added to the main compartment of the unit (mf) made from a 50 ml glass stoppered flask The flask contained 3 ml of a 10 per cent trichloracetic acid The content of the flask was stirred magnetically for 2 hours and the carbon dioxide evolved was captured into 0 3 ml of Hyamine 10X contained in a small cup in a side tube (st)

In the other series of experiments rats weighing between 150 and 160 g were used In this group one animal was killed every 15 min of an exposure whose final length was 120 min and the CO content in the acid labile the acid soluble the protein the lipid and the nucleic acid tissue fraction was determined

All animals were anaesthetized with Nembutal (3—4 mg/100 g b w) tracheotomized and allowed to breathe spontaneously The animals were left undisturbed for 15 min before they were exposed to the radioactive gas mixture In experiments with long exposure times the animals sometimes began to recover from the anesthesia and reacted to painful stimuli In such cases an additional dose of Nembutal was given (1 mg/100 g b w) All rats with respiratory depression or with irregular breathing were rejected During the exposure period the head of the rat was secured with a head holder while the body rested on a metal box which could be heated with a lamp so as the minimize heat losses

After each exposure period the rat was disconnected from the radioactive gas mixture and the head was quickly frozen by immersion into liquid nitrogen When the head was completely frozen it was sawed into slices about 5 mm in thickness using a small hand saw with exchangeable blades In order to prevent any thawing of the tissue during the preparation the saw and the the head of the animal were intermittently immersed into liquid nitrogen The brain

tissue was then separated from the skull by pressing with the point of a chilled surgical blade at the margin of the bony coverings This procedure usually left the dura intact but it could easily be removed with the help of a surgical blade Two of the separated brain tissue slices were usually used to prepare cortical samples which were split off 1—2 mm from the surface of the frozen tissue

Each animal was exposed to a constant concentration of CO in the inspired air This was achieved by the following procedure (Fig 1 A) The radioactive stock solution was prepared from 1 0 mc Na_2 CO (The Radiochemical Centre Amersham England) which was rinsed down into a 10 ml volumetric flask and filled up to the mark with distilled water For each experiment 2 0 ml (200 μc) of this solution was added to a 50 ml diffusion flask (df) which also contained a magnetic stirrer The diffusion flask (df) was then connected to a 12 litre delivery flask (def) via a ground glass joint The latter as well as the stopper of the diffusion flask was lubricated with syrupy phosphoric acid The delivery flask (def) had a rubber stopper (rs) which admitted the glass tube from the diffusion flask (df) an inlet tube from a reservoir flask (rf) and finally the spindle from a small electrical motor (fm) used for driving a fan inside the delivery flask In addition to the rubber stopper (rs) the delivery flask (def) was equipped with a Geiger Muller tube (gm) snugly fitted into a burr hole in the wall of the delivery flask (def) and with an outlet tube connecting the flask with a fan The spindle of the electrical motor was equipped with a coaxial cup turned upside down which revolved in a larger cup filled with glycerol so as to prevent leakage of the radioactive gas mixture

After the diffusion flask (df) had been connected with the delivery flask (def) 2 ml of a 0 1 N H_2SO_4 solution was injected through a rubber stopper fitted into a side tube of the diffusion flask (df) and the content of the diffusion flask was stirred magnetically The rate of diffusion of CO from the diffusion flask (df) into the delivery flask (def) was measured in preliminary experiments the CO activity within the delivery flask (def) being measured with a Geiger Muller tube connected to a rate meter and an ink writer It was found that a constant activity was obtained after a diffusion time of about 2 5 hrs and that the activity remained unchanged even after 7 hrs In each subsequent experiment a diffusion time of 4 hrs was allowed for before the rat was connected to the gas supply

After completed diffusion the clamp on the outlet tube from the delivery flask (def) was opened together with another clamp on the inlet tube (c) from the reservoir flask (rf) The latter which was placed about 1 5 m above the delivery flask (def) contained glycerol which has a low solubility for carbon dioxide (International Critical Tables 1928) The rate of flow of glycerol was controlled by adjusting the clamp (c) In such a way the radioactive gas mixture was forced to flow in the outlet rubber tubing at a rate which could easily be adjusted Each rat was then connected to the radioactive source by joining the straight tracheal cannula with a T branch on the outlet tube

For the determination of the amount of CO in *the acid-labile carbon dioxide fraction* of the brain tissue samples the acid labile carbon dioxide was diffused over into a 1 M methanolic solution of a quaternary organic base (Hyamine 10-X Rohm and Haas, Philadelphia) according to Radin (1958) Before the tissue samples were transferred to the diffusion units (Fig 1 B) they were ground to a fine powder in a mortar which was kept filled with liquid nitrogen Aliquots of the powdered tissue (100—700 mg) were then added to the main compartments (mf) of the previously weighed diffusion units each of which contained 3 ml of a 10 per cent trichloracetic acid solution The side tube (st) of each diffusion unit contained a small glass cup of about 0 6 ml capacity holding 0.3 ml Hyamin 10-X. After the frozen tissue had been added and the diffusion units stoppered the contents of the main compartments were stirred magnetically for ? hours Preliminary experiments had shown that this time period was sufficient for the absorption of more than 98 per cent of the CO into the Hyamine 10-X solution

The amount of tissue added to the diffusion units was weighed by difference on an analytical balance The CO activity in the samples was then determined with a liquid scintillation technique The scintillation mixture contained 4 g of 2 5-diphenyloxazole (PPO) and 0 1 g of 1 4-di [2 (5-phenyloxazole)] benzene (POPOP) per litre of toluene (p.a) The glass cups containing the Hyamine 10-X were transferred to the counting vials and 5 ml of the scintillation mixture was added to each vial The vials were then refrigerated overnight and the samples subsequently counted in a Panax liquid scintillation equipment Before counting the glass cups were carefully emptied and removed The samples were counted for 10^4 seconds with an E.H.T of 1400 V and a discriminating voltage of 10 V A blank was run for each experiment For this purpose 0.2 ml of a 0 01 M Na CO solution was added to the trichloracetic acid in a diffusion unit and the sample was then treated identically with the tissue sample The blank

count was subtracted from the tissue count and the latter was then calculated as the number of counts per gram of tissue

For the determination of the tissue $^{14}CO_2$ activity in the second type of experiments employing rats weighing 150—160 g the following procedure was adopted After the tissue had been crushed to a fine powder it was divided into 3 portions One was transferred into the diffusion units for the determination of *the acid-labile CO_2* as described above The second aliquot (200—400 mg) was transferred to a stoppered test tube containing 2 ml of Hyamine 10 X and was then digested in a water bath at 65 for 9—12 hours (Dulcino *et al* 1963) This sample represented *the total amount of* $^{14}CO_2$ present in the tissue The third aliquot (200—400 mg) was homogenized in the cold (ice bath) in 5 % trichloracetic acid in centrifuge tubes with specially fitted Perspex pestles The precipitate resulting from centrifugation was then washed twice with 5 % trichloracetic acid and the washings were combined to yield *the acid soluble fraction* *The lipids* were extracted 3 times from the washed precipitate using a 2 1 mixture of chloroform methanol *The nucleic acids* were extracted at 90 in 5 % trichloracetic acid and the residue — *the crude protein fraction* — was washed with acetone and ether and air-dried Before further handling of the samples the acid-soluble and the nucleic acid fractions were extracted several times with ether to remove most of the trichloracetic acid These two fractions as well as the lipid fraction were then taken down to a small volume in a stream of air at 50 —60 To each sample including the protein fraction was then added 2 ml of Hyamine 10-X the test tubes were stoppered and the samples were digested for 6—12 hours at 65

The different samples were left for digestion until clear and only slightly coloured solutions remained In the whole tissue sample and in the protein sample a few tiny strands of tissue usually were left undigested They were however so small that their quantitative influence on the results should be negligible The samples could not be left for digestion for more prolonged time periods since the solutions then turned brown resulting in a very low counting efficiency The same thing could occur if the temperature of digestion was increased above that recommended

When the samples had been digested they were diluted with 2 4 dioxane up to a final volume of 10 ml Aliquots (2 ml) of the resulting solutions were then added to the counting vials together with 5 ml of scintillation mixture The latter was prepared with PPO POPOP and naphtalene in toluene according to Dulcino *et al* (1963) The vials were then refrigerated until counting It was found that the samples had to be left refrigerated for about 24 hours in order that consistent count rates should be obtained with the protein rich samples Optimal counting conditions were found to be obtained with an E H T of 1 350 V and a discriminating voltage of 15 V The samples were counted for at least 10^4 sec and the background was corrected for by passing a blank sample through the whole procedure An internal standardization was also used in that aliquots of a standardized hexadecane 1 C-14 solution (The Radiochemical Centre Amersham England) was added to each sample with subsequent recounting The count rates were then expressed as the number of disintegrations per gram of tissue and minute

The 2 4 dioxane used for diluting the samples was refluxed 0 5 hours over stannous chloride shaken up with potassium hydroxide refluxed 0 5 hours over sodium and then distilled off the sodium The toluene (p a) was used as obtained commercially

Results

A *The equilibration of the administered* CO_2 *with the acid labile carbon dioxide of brain tissue*

In a previous publication results were presented to show that inspired $^{14}CO_2$ is rapidly incorporated into the acid labile CO_2 fraction of the brain equilibrium being reached in about 60 min (Siesjo 1963) In order to allow exposure periods up to 120 min rats weighing between 150 and 160 g were then used for the longer exposure times In the present material however which comprises animals from the older material as well as new ones all rats weighed around 200 g (190—210 g The effective volume of the delivery flask (see Fig 1) i e the volume of gas which could be displaced by the glycerol was slightly more than 11 litres With the long exposure times (90 min or less) the rate of flow of gas in the outlet tube was thus about 125 ml per minute This volume exceeds by about 20 per cent the tidal volume of rats of the present size (Drorbaugh 1960)

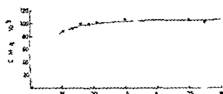


Fig 2

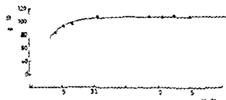


Fig 3

Fig 2 (left) The rate of equilibration between inspired CO and the diffusible acid labile carbon dioxide fraction of brain tissue The radioactivity is expressed as the number of counts per gram of tissue per minute Each point represents one experiment The curve drawn through the points follows the function y = 1 05 × 10 (1—0 614 e$^{-0\ 28t}$—0 518 e $^{0\ 075}$)

Fig 3 (right) The rate of equilibration between inspired CO and the diffusible acid labile CO fraction of cortical tissue The curve drawn through the points follows the function y = 1 08 10 (1—0 581 e$^{0\ 8t}$—0 679 e$^{0\ 10\ t}$) Note that the diffusible carbon dioxide fraction of cortical tissue comes into equilibrium with the CO at a faster rate than does that of the whole brain

The specific activity of the radioactive CO_2 released into the system was so high that the resulting CO_2 tension in the gas phase never exceeded 1 mm Hg This tension could be assumed to be without detectable physiological effects

The rate of equilibration between the inspired ^{14}CO and the acid labile fraction of the tissue is illustrated in Fig 1 where the radioactivity of the brain tissue samples expressed as number of counts per gram of tissue per minute has been plotted against the time of exposure It is seen that with the type of rats used an apparent equilibrium was reached after about 45 min after which time no significant increase occurred in the activity

The corresponding curve for the cortical tissue is shown in Fig 2 If this curve is superimposed upon that of Fig 1 it can be seen that although the absolute figures at equilibrium are comparable to those for the whole brain the rate of uptake of CO_2 appears to be somewhat more rapid in the cortical tissue

An attempt was made to find equations which would approximately describe the rate of uptake of CO_2 as illustrated in Fig 1 and 2 It was found that no single exponential function fitted the experimental points The curve was therefore analysed according to the procedure described by Solomon (1949) In order to increase the accuracy however the logarithm of the experimental values in the last part of the curve (log y) was plotted against time (t) and the slope of the resulting curve was determined in a number of points with the help of a dervometer (Arner 1964) When the values for dy/dt were plotted against the corresponding values for y a straight line could be drawn through a number of points The slope of this line gave the coefficient k in the equation below and by inserting this value into the equation dy dt c_2 k_2 e ^{k} the corresponding value for c was obtained The exponential term resulting was then subtracted from the original curve in the usual way (Solomon 1949) yielding a second exponential term No attempt was made to derive a third exponential function from the experimental curve

The rate of uptake of CO into the acid labile CO fraction of brain tissue could be approximately described by the equation y = 1 05 × 10 (1—0 614 e ^{8} — 0 518 e $^{0\ 0\ 5}$ where y is expressed as counts min g of tissue It can be seen from the equation

TABLE I Incorporation of ${}^{14}CO$ (dpm/g of tissue) into the acid labile (diffusible) the acid soluble the protein, the lipid and the nucleic acid tissue fractions of brain tissue The last column denotes the recovery of the ${}^{14}CO$ in the different fractions as compared to the total ${}^{14}CO_2$ measured in the unfractionated tissue

Exposure time in min	Disintegrations/min/g of tissue T Total	D Diffusible	AS Acid Soluble	P Protein	L Lipid	NA Nucl Acid	D+AS+P+L+NA/T
15	110 400	99 200	7 300	1 800	500	500	99 1
30	153 600	128 700	16 800	2 000	500	600	96 7
45	172 000	141 600	27 400	2 300	600	1 200	97 2
60	196 800	157 200	30 600	2 900	600	1 100	97 8
75	195 000	151 800	34 100	3 200	800	1 800	98 3
90	222 000	164 100	53 400	5 200	2 100	1 600	102 0
105	231 600	162 600	54 300	4 200	1 400	1 400	96 7
120	255 500	164 400	75 000	6 600	2 300	2 600	98 2

that the sum of the constants c_1 and c_2 exceeds unity implying that the curve starts with a certain time lag

A similar analysis was performed of the rate of incorporation of ${}^{14}CO_2$ into the cortical tissue This analysis could not be performed with the same accuracy due to the scatter of the experimental values An approximate fit was given by the equation $v = 1\,08 \times 10\ (1-0\,584\,e^{-0\,28t} - 0\,672\,e^{-0\,105t})$

A comparison between the two equations indicated that the incorporation into the acid labile fraction of cortical tissue did occur at a faster rate than that into whole brain tissue and that the difference was due to the second slow exponential term (see discussion)

B *The equilibration of the administered ${}^{14}CO_2$ with the acid labile the acid soluble the protein the lipid and the nucleic acid tissue fractions* In this series of experiments smaller rats were used (see Methods) and it became apparent that with these rats the time needed for an equilibrium to be reached between the administered ${}^{14}CO_2$ gas mixture and the acid labile CO_2 fraction of the brain was slightly longer (*cf* Siesjo 1963) The emphasis in this series was however laid on a comparison between the rate of incorporation of ${}^{14}CO$ into different tissue fractions The relation between these rates are illustrated in Table I where the counting rates obtained with the different fractions have been compiled together with the per cent recovery

The total ${}^{14}CO_2$ activity in the tissue including the acid labile and the different organic fractions increased roughly exponentially during the first 60 min after which time the increase was approximately linear The exponential increase during the first hour was given by the acid labile fraction (CO_2 H_2CO_3 HCO_3^-) which reached an apparent equilibrium during that time while the sum of labelling of the different organic fractions (As + P + L + NA) was labelled at a linear rate during the whole exposure period

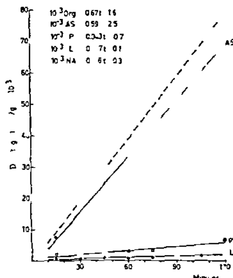


Fig 4 The rate of equilibration between inspired CO_2 and the organic fractions of the tissue The individual points for the total CO_2 incorporation into organic fractions (Org broken line) have been omitted to avoid obscuring those representing the incorporation into the acid soluble fraction Also the values for the CO recovered in the nucleic acid fraction (NA) have been omitted since they correspond very closely to those for the lipid fraction (L)

The relation between the rate of incorporation of CO_2 into the different organic fractions is illustrated in Fig 4 where the equations for the straight lines have been calculated in order to facilitate comparisons The curve with the steepest slope represents the CO_2 activity in all the organic fractions If this is compared to the total CO_2 activity in the tissue (all the organic fractions + the diffusible acid labile fraction) it is found that after 15 30 60 and 120 min the organically fixed CO_2 in the tissue amounts to 8 13 21 and 32 per cent of the total activity respectively The main part of the organic CO_2 was recovered in the acid soluble fraction (about 85 per cent) while most of the remaining activity was found in the protein fraction (8—9 per cent) The lipid fraction and the nucleic acid fraction were equally labelled but the activity in these fractions was too low to allow any quantitative comparisons with the other fractions Thus in none of these fractions did the CO_2 activity ever amount to one per cent of the total tissue activity It should be pointed out however that the radioactivity in these fractions appeared to increase steadily with increasing time of exposure

Discussion

As a first approximation it might be assumed that the laws governing the uptake of inert gases should also apply to CO_2 According to these laws which have been competently described (Morales and Smith 1948 Jones 1950 Kety 1951 *cf* also Severinghaus 1964) the rate of uptake of a gas in a tissue is determined by 3 main factors the lung ventilation the solubility of the gas in the blood and the blood flow to the tissue However even if it could be assumed that CO_2 fits qualitatively into these schemes its peculiar characteristics make it adventurous to apply any quantitative relations formulated for other gases to CO_2 equilibria Thus CO_2 is not only dissolved in the blood and in the tissue but also chemically bound which implies that its absorption in the

different phases does not follow Henry s law rather different carbon dioxide absorption curves Moreover the gas is not physiologically inert but influences such parameters as the lung ventilation and the blood flow to the brain This is to say that the accumulation and the elimination of CO leads to physiological changes which in their turn influence the rate accumulation and the rate of elimination respectively (*cf* Landau 1963) Finally the time taken for the diffusion of the gas in the tissue cannot immediately be neglected since the rate-determining diffusion coefficient incorporates a factor for the binding of CO in the tissue (Siesjo and Thews 1962)

It is clear that some of the difficulties encountered when studying the kinetics of CO transients may be avoided by using the radioactive compound Thus the concentration of the administered gas can be kept so low that the physiological parameters do not change appreciably The curve for the incorporation of CO into the acid labile CO_2 of the tissue should then illustrate the basic rate of accumulation Any moderate increase of the CO_2 concentration in the inspired gas mixture should by virtue of its effect upon the lung ventilation and upon the tissue blood flow tend to increase to rate of incorporation

There is however a complication to this simple scheme which concerns the equilibrium between the inspired CO_2 and the total carbon dioxide content of the tissue Thus the CO coming to the tissue does not only exchange with the bicarbonate pool of the tissue but also with the carboxyl groups of free and protein bound organic acids



R COOH

The incorporation of CO into organic compounds in the brain proceeds at a rate which is linear with time during the period studied presently This means that the CO concentration should be constantly below its equilibrium value in the present experiments and that the acid labile carbon dioxide fraction has not reached a true equilibrium during the 90 min studied in the first series of experiments However since the amount of CO which is incorporated per minute into organic compounds is less than 0 5 per cent of the total acid labile CO at equilibrium and since the rate of diffusion of CO is high (Siesjo and Thews 1962) the CO activity in the present experiments should be rather close to the true equilibrium values

The present work was undertaken to investigate the possible presence of an active transport of hydrogen ions between brain tissue and blood plasma Such a transport may be approached from kinetic and from physicochemical angles In the present context only the kinetic approach will be touched upon while the physico-chemical arguments will be left for further treatment (Siesjo 1964) The present work has shown that if rats are exposed to a gas mixture with radioactive CO_2 the diffusibly carbon dioxide fraction of the brain (CO_2 H CO and HCO_3) comes into an apparent equilibrium with the inspired CO_2 within an hour or less According to available theory (see above) this equilibrium would be reached much quicker if the CO tension of the inspired gas mixture was increased However in the work of Nichols (1958) and in experiments from the laboratory (Siesjo 1964) the apparent equilibrium time was not shorter than the present one but 3 times as long (see introduction) The discrepancy between the results obtained in the two studies may be tentatively explained in a number of ways (see Siesjo 1964) and one possible explanation considers an active transport of hydrogen ions or of bicarbonate ions across the blood brain barrier

Thus if carbon dioxide is administered a new mean tissue CO_2 tension will be reached in less than 20 min indicating that at that time the tissue concentration of dissolved CO and of carbonic acid will be reasonably constant in all tissue compartments This means that any further increase in the total CO content of the tissue must imply that bicarbonate ions are transferred into the tissue passively or actively or that hydrogen ions are carried the opposite way Experiments have shown that there are no significant passive fluxes of these ions between the brain and the blood plasma (Siesjo 1964 b) but it remains to be conclusively shown that the responsible process is an active transport activated by the acidogenic load and not any one of a number of other hypothetical processes which can explain the disappearance of hydrogen ions from a metabolizing system

Apart from their implication for the study of acti e transport mechanisms the present results have a bearing also on the elucidation of carbon dioxide transients A quantita ti e interpretation of such transients in terms of rate limiting factors and compartments would require more experimental data than provided here However it is tempting to assume that the fast and slow components in the present curves (Fig 2 and 3) are dominated by the lung washout and the tissue blood respectively and that the faster equilibrium in cortical tissue is due to the higher blood flow (see Kety 1950)

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From the Department of Pharmacology University of Goteborg Sweden

5 Hydroxyindoleacetic Acid of Rabbit Spinal Cord Normally and after Transection

By

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Abstract

Andén N E T Magnusson B E Roos and B Werdinius *5-Hydroxyindoleacetic acid of rabbit spinal cord normally and after transection* Acta physiol scand 1965 64 193—196 — The normal spinal cord of rabbits was found to contain 0 20 µg/g 5-hydroxyindoleacetic acid in the part cranially to Th4—Th6 Caudally the concentration was 71 per cent of that cranially After transection at this level there was no significant change in the cranial part when analyzed as a whole Caudally to the lesion the 5-hydroxyindoleacetic acid showed a substantial drop during the first 2 days and thereafter a gradual fall It had disappeared almost completely after 10 days The 5 hydroxytryptamine determined in the same rabbits was unchanged after 2 days was considerably reduced after 5 days and had almost completely disappeared after 10 days It is concluded that the level of 5 hydroxyindoleacetic acid better than that of 5-hydroxytryptamine mirrors the metabolic rate of 5-hydroxytryptamine

Recent biochemical and histochemical investigations have shown that the 5-hydroxytryptamine (5 HT) in the spinal cord is localized in descending nerve tracts (Carlsson Magnusson and Rosengren 1963 Carlsson *et al* 1964) The 5-HT is highly concentrated in varicosities of axon terminals (Carlsson *et al* 1964) belonging to neurons the cell bodies of which are situated in the lower brain stem (Dahlstrom and Fuxe 1964 b) Electrical stimulation of these descending pathways results in release and increased synthesis of 5 HT Anden *et al* 1964 a) Administration of the 5 HT precursor 5-hydroxytryptophan produces profound functional changes in the spinal cord (Carlsson Magnusson and Rosengren 1963 Andén Jukes and Lundberg 1964 Andén *et al* 1964 c Andén Carlsson and Hillarp 1964) an effect due to the accumulation of 5-HT there (Anden Jukes and Lundberg 1964) The findings that the 5-HT in the spinal cord is released by nerve activity and has functional effects strongly indicate that 5-HT serves as a humoral transmitter there All these investigations have prompted the present study on the effect of transection on the spinal cord levels of 5-hydroxyindole acetic acid (5-HIAA the main metabolite of 5-HT

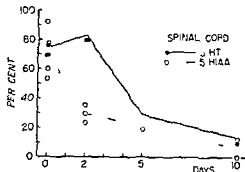


Fig 1 The levels of 5 hydroxyindoleacetic acid (5 HIAA) and 5 hydroxytryptamine (5 HT) of the caudal part of the rabbit spinal cord in per cent of those of the cranial part at various intervals after transection Each symbol represents one single determination

Material and methods

Adult rabbits of both sexes weighing 1 5—3 0 kg were used Under pentobarbital sodium anesthesia (about 30 mg/kg i v) the spinal cord was transected at Th4—Th6 Postoperatively care was taken to prevent hypothermia residual urine decubitus and wound infections After various intervals of time the animals were sacrificed by air embolism The spinal cords were freed from the meninges and nerve roots The pieces cranially and caudally to the lesion from four to seven rabbits were rapidly pooled and homogenized in 0 1 N HCl (3 ml per g tissue) The proteins were precipitated with 10 % $ZnSO_4$ 7H_2O (1 ml per g tissue) plus N NaOH (0 5 ml per g tissue) The 5 HIAA and 5 HT of the same extract were separated purified and concentrated by means of an organic solvent extraction procedure and determined spectrophotofluorimetrically in 3 N HCl (Roos 1962 Roos and Werdinius 1962) The fluorescence spectra were always recorded The recoveries of 2 μg 5 HT and 2 μg 5 HIAA to an aliquot of a homogenate were found to be about 70 and 60 per cent respectively when no allowance for partition was made All values were corrected for the recovery Normal values were obtained from rabbits not operated upon

Results

Cranially to the transection site the spinal cords of the non operated rabbits were found to contain on the average 0 33 μg/g 5 HT (S E = 0 041 4 expts) and 0 20 μg/g 5 HIAA (S E = 0 031 4 expts) The levels of these compounds in the cranial part of the spinal cord after the operation were for 5 HT 0 31 μg/g (S E = 0 019 7 expts) and for 5 HIAA 0 22 μg/g (S E = 0 021 7 expts) Apparently the transection did not change the concentrations of these substances significantly in the cranial portion of the spinal cord when analyzed as a whole For this reason it was possible to give the levels of 5 HT and 5 HIAA in the part caudally to the lesion as the percentage of those occurring cranially (Fig 1)

Caudally to the transection site of the non operated rabbits the concentrations of 5 HT and 5 HIAA were on the average 75 (S E 3 5 4 expts) and 71 (S E = 8 8 4 expts) per cent respectively of those cranially to it During the first 2 postoperative days the 5-HT in the caudal portion was approximately unchanged It showed a substantial drop between 2 and 5 days and had almost completely disappeared after 10 days Also the 5 HIAA disappeared from the caudal part of the spinal cord after transection The time course of the disappearance of 5 HIAA was however somewhat different from that of 5 HT In the first 2 days after the operation the 5 HIAA content of the caudal part was reduced from 71 to 23 per cent of that of the cranial one Thereafter there was a gradual drop of the 5 HIAA After 10 days the caudal part was almost completely depleted of 5-HIAA

Discussion

In normal rabbits the proportion between the concentrations of 5 HT and 5 HIAA is about the same in the brain and in the spinal cord The levels are however 2—3 times higher in the brain (Roos and Werdinius 1962 Andén Roos and Werdinius 1963 and unpublished results)

The time courses of the disappearance of monoamines in the spinal cord caudally to a transection have been investigated earlier (Andén *et al* 1964 b) It was found that the depletion of 5-HT is most rapid between the fifth and seventh postoperative day whereas that of noradrenaline is almost finished after 5 days The drop of 5-HT appeared somewhat earlier in the present study which may possibly be due to individual differences It has earlier been observed that the noradrenaline of the rabbit iris disappears at different rates in different animals after excision of the superior cervical ganglion (Andén *et al* 1964 b) However individual differences cannot be the reason for the finding that the concentration of 5 HIAA in the caudal part was considerably reduced at the second postoperative day when that of 5 HT was about unchanged since the values were obtained from the same rabbits Probably the level of 5-HIAA better than that of 5 HT mirrors the retardation of the 5-HT metabolism after section of the descending 5 HT pathways in the spinal cord

In the intact spinal cord the 5 HIAA may be formed from the 5-HT released from the nerve granules into the synaptic gap by nerve activity Since this release should cease immediately after section of the descending 5-HT nerves in the spinal cord this effect may be the cause of the rapid disappearance of the 5 HIAA The formation of 5-HIAA can however probably occur in other ways For example reserpine has been found to produce a long lasting rise of the 5-HIAA in the brain (Ashcroft and Sharman 1962 Roos and Werdinius 1962 Andén Roos and Werdinius 1963) It can be assumed that reserpine simultaneously reduces the nerve impulse induced release of 5 HT like that of catecholamines from the nerve endings (Carlsson *et al* 1957 Carlsson 1964) by blocking the incorporation of monoamines in the granules (Kirshner 1962 Carlsson Hillarp and Waldeck 1963 Euler and Lishajko 1963) Therefore the conversion of 5 HT to 5 HIAA can take place intracellularly before the amine is incorporated in the granules The elevated level of 5-HIAA after reserpine treatment may also be due to an enhanced synthesis of 5-HT (Andén Roos and Werdinius 1963) It has been found that the 5 HT synthesis in the spinal cord is influenced by the activity of the descending nerve tracts (Andén *et al* 1964 a) The disruption of the impulse flow by cutting the spinal cord should thus cause a diminished synthesis of 5 HT and 5 HIAA This may be responsible for the drop of the 5 HIAA within the first 2 days after the transection although the 5 HT stores are still intact The 5 HIAA remaining after 2 and 5 days may reflect the slow metabolism of 5 HT occurring after denervation It has been observed that the 5 HT metabolism in the caudal part of the spinal cord does not immediately cease after a transection as inhibition of the monoamine oxidase causes a slow accumulation of 5-HT (Dahlström and Fuxe 1964 a) with the development of 5-HT effects (Andén Jukes and Lundberg 1964) The approximately simultaneous disappearances of 5-HT and the last of 5-HIAA may occur when the 5 HT terminals are degenerating Anyhow the earlier fall of 5-HIAA after spinal cord transection seems to show that the acid better than the amine reflects the turnover rate of 5 HT

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Distribution of Monoamines and Dihydroxyphenylalanine Decarboxylase Activity in the Spinal Cord

By

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Abstract

Anden N E. *Distribution of monoamines and dihydroxyphenyl lalanine decarboxylase activity in the spinal cord* Acta physiol scand 1965 *64* 197—203 — The contents of noradrenaline 5-hydroxytryptamine and DOPA decarboxylase activity at different levels of the rabbit and cat spinal cord have been investigated The concentrations in the various parts differed only slightly and roughly parallelled the relative amounts of grey matter There was more 5-hydroxytryptamine than noradrenaline in all locations usually 2—4 times The distribution in the thoracic cord of cows has also been investigated The concentrations of both amines were highest in the lateral horns lower in the anterior and posterior horns and much lower in the white matter The DOPA decarboxylase activity was lower in the lateral horns than in the white matter and the other horn No dopamine was normally found in the spinal cord

The presence of noradrenaline (NA) in the spinal cord was first demonstrated by Euler (1946 1950) and Vogt (1954) and that of 5-hydroxytryptamine (5-HT) by Amin *et al* 1954) *However until recently nothing was known about the cellular localization and function of these amines in the spinal cord It was possible that they did not occur* in central neurons but in vasomotor nerves blood platelets or glia cells Now it has been proved that both amines are localized in descending nerves of the spinal cord Magnusson and Rosengren 1963 Carlsson Magnusson and Rosengren 1963 Carlsson *et al* 1964 Andén *et al* 1964 b) and probably serve as synaptic transmitters (Carlsson *et al* 1964 Andén *et al* 1963 1964 a and c Andén Jukes and Lundberg 1964 Andén Carlsson and Hillarp 1964) These findings prompted studies on the distribution of NA *and 5-HT in the spinal cord Such data will be presented and discussed below In* addition the activity of L-3 4-dihydroxyphenylalanine (DOPA) decarboxylase was determined in different parts of the spinal cord

Material and methods

Adult cats and rabbits were used for the determination of the concentrations of dopamine (DA), NA and 5-HT at various levels of the spinal cord. The activity of the cord DOPA decarboxylase determined *in vitro* was also mapped out in these two species. The assays of the catecholamines, 5-HT and DOPA decarboxylase activity were performed on different animals. The cats were sacrificed by bleeding under N_2O anesthesia. In the 5-HT experiments the rabbits were anesthetized with pentobarbital sodium (20—30 mg/kg i. v.) and bled to death from a carotid artery at the same time as they received an infusion of oxygenated Ringer's solution at 37° C into an external jugular vein. This procedure eliminated contamination by blood platelet 5-HT. In the other experiments the rabbits were sacrificed by air embolism. The spinal canal was opened, the dura removed and the spinal cord taken out as soon as possible. The spinal roots were used to identify the segments. The 1—2 cm of the cord cranial and caudal to the cervical enlargement and the four cranial lumbar segments were removed and discarded in order to permit comparison of representative pieces. The pia mater and nerve roots were stripped off. The NA was determined spectrophotofluorometrically after ion exchange chromatography and oxidation by the procedure of Bertler, Carlsson and Rosengren (1958) or, when the NA amounts were small, by the modification of Häggendal (1963). The 5-HT was determined spectrophotofluorometrically in 3 N HCl after ion exchange chromatography according to Bertler (1961 a) except that the tissue residue was reextracted with about 3 ml 0.4 N perchloric acid per g tissue (Carlsson and Lindqvist 1962). The activation and fluorescence spectra of the apparent and authentic 5-HT were recorded. The DOPA decarboxylase was extracted with phosphate buffer and incubated with L-DOPA as described by Bertler and Rosengren (1959 a). The DOPA decarboxylase activity was determined also *in vivo* in cats. These cats, which were decerebrated and used partly for other purposes, were given 67 or 100 mg/kg L-DOPA i. v. about one hour before sacrifice. The DA formed *in vitro* as well as *in vivo* was estimated spectrophotofluorometrically after ion exchange chromatography and oxidation (Carlsson and Waldeck 1958, Carlsson and Lindqvist 1962).

The thoracic cords of the cows were received from the slaughter house. The cows were sacrificed by a shot into the brain. About one hour elapsed before the spinal cord was taken out and cooled with ice. The grey columns of the thoracic cord with the surrounding white matter were obtained by punching out the requisite parts with a steel punch fitted with a steel plunger. During the preparation, which usually lasted about three hours, the cords were kept ice cooled. The losses during this procedure were controlled by taking a piece of the whole spinal cord which was extracted at once. The catecholamines, 5-HT and DOPA decarboxylase activity *in vitro* were determined as described above.

Results

Distribution of amines in the spinal cord. At all levels of the rabbit and cat spinal cord NA and 5-HT were present (Table I). No DA was found normally. The distributions of NA and 5-HT were rather even in the rabbit spinal cord. The concentrations of both substances were, however, about twice as high in the conus medullaris as in the cranial parts. At all levels there was 2—4 times more 5-HT than NA. The concentrations of NA and 5-HT in the cat spinal cord were very similar to those found in the rabbits cranial to the lumbar enlargement. In the cat lumbar enlargement (L5—S1) the concentrations of both monamines were about twice as high as in the cranial regions. The conus medullaris of the cat contained much 5-HT, about five times more than of NA. Different animals showed large variations of the concentrations but conspicuously constant distributions. There was an almost complete disappearance of both NA and 5-HT in all parts of the spinal cord about 16 hrs after reserpine treatment (cats 5 mg/kg i. p., rabbits 2 mg/kg i. v.).

The concentrations of NA and 5-HT in the anterior and posterior parts of the cat lumbar enlargement were investigated in 2 expts. The anterior half was found to

TABLE I Distribution of noradrenaline (NA) and 5-hydroxytryptamine (5 HT) in the spinal cord of cats and rabbits The figures in brackets in the headings refer to number of animals The values are in μg/g tissue and are means with the range in brackets

	Rabbit		Cat	
	NA (2)	5-HT (2)	NA (6)	5 HT (6)
C1—C3	0 09 (0 03—0 10)	0 25 (0 ?1—0 ?9)	0 08 (0 04—0 12)	0 18 (0 07—0 ?8)
Cervical enlargement	0 09 (0 03—0 10)	0 34 (0 33—0 35)	0 12 (0 07—0 14)	0 3[illegible] (0 24—0 4)
Th3—L1	0 07 (0 06—0 07)	0 2? (0 21—0 23)	0 11 (0 09—0 13)	0 25 (0 15—0 37)
Lumbar enlargement	0 14 (0 10—0 17)	0 34 (0 ?4—0 34)	0 ?3 (0 13—0 34)	0 74 (0 34—1 12)
Conus medullaris	0 ?6 (0 23—0 ?9)	0 59 (0 33—0 ?4)	0 ?8 (0 17—0 43)	1 41 (0 —1.9

TABLE II Distribution of DOPA decarboxylase activity in the spinal cord of cats and rabbit The figures in brackets in the headings refer to number of animals. The values are expressed in μg dopamine per g tissue formed in vitro after 45 min incubation with L DOPA or in vivo after injection of L DOPA The values are means with the range in brackets

	Rabbit	Cat	
	In vitro (3)	In vitro 2	In vivo 6
C1—C3	40 3 —45)	26 (24—?	1 9 (1 3—3 4
Cervical enlargement	40 31 47	24 0—?)	? [illegible] 1 3—4 3
Th3—L1	33 31—43	? (?3—30	1 9 1 0—3 4
Lumbar enlargement	41 (35— 0)	43 (39—47	8 1 6—4
Conus medullaris	54 3[illegible]—[illegible]9	7 6[illegible]—87	8 0 4 —1

TABLE III Distribution of noradrenaline (NA) 5-hydroxytryptamine (5-HT) and DOPA decarboxylase activity in the thoracic cord of cows The figures in brackets in the headings refer to number of determinations The values of NA and 5-HT are in µg/g tissue and the DOPA decarboxylase activity is expressed as the formation of dopamine in µg/g tissue after 45 min incubation with L-DOPA The values are means with the range in brackets

	NA (2)	5-HT (2)	DOPA decarboxylase activity (2)
Lateral horn	0.23 (0.14–0.32)	0.50 (0.43–0.56)	83 (65–100)
Anterior horn	0.05 (0.05–0.05)	0.14 (0.12–0.16)	350 (330–360)
Posterior horn	0.11 (0.08–0.13)	0.14 (0.14–0.14)	220 (200–230)
White matter	0.01	0.02*	270 (240–300)
Grey + white matter before dissection	0.07	0.08	220 (200–240)
after dissection	0.02	0.04	2[illegible]0 (250–290)

* Single determination

contain 0.12 µg/g NA and 0.66 µg/g 5-HT whereas the corresponding values of the posterior half were 0.14 µg/g and 0.67 µg/g respectively

Distribution of DOPA decarboxylase activity in the spinal cord The distribution of DOPA decarboxylase activity in the spinal cord of rabbits and cats (Table II) was very similar to those of the monoamines In the rabbit cord the activity when determined *in vitro* was rather evenly distributed but was somewhat higher in the conus medullaris than in the other regions In the cat spinal cord the *in vitro* activities of the parts cranial to the lumbar enlargement were somewhat lower than those of the corresponding parts in rabbits The lumbar enlargement contained more than the cranial portions and the conus medullaris still more in agreement with the higher monoamine concentrations in these regions Since no DA was found normally in the cat spinal cord and the NA formation was negligible the DA concentration after injection of L-DOPA should reflect the enzyme activity *in vivo* As seen in Table II the activities obtained *in vitro* and *in vivo* agreed satisfactorily

Distribution of amines and DOPA decarboxylase activity in the thoracic cord The distributions of NA and 5-HT in the cow thoracic cord are presented in Table III Like in the cat and rabbit spinal cords no DA was observed The highest concentrations of NA and 5-HT occurred in the lateral horns These substances were present also in the

anterior and posterior horns but in lower concentrations The posterior horns included also the intermedio-medial nucleus and the substantia grisea centralis (Rexed 1954) In the white matter the two amines occurred in very low concentrations In all places there was more 5-HT than NA

The distribution of the DOPA decarboxylase activity determined *in vitro* was apparently different from those of the amines in the thoracic cord (Table III) The enzyme activity was high in the white matter as well as in the anterior and posterior horns whereas it was much lower in the lateral horns

Discussion

In all locations of the brain where 5 HT occurs there is also a catecholamine usually NA but DA in *e g* the caudate nucleus and the putamen (Bertler 1961 b) The same holds true for the spinal cord with NA as the only catecholamine found normally The NA and 5 HT in the spinal cord are however much more evenly distributed than in the brain The proportion between the two amines is also strikingly constant at the different levels of the spinal cord In fact the small differences between the concentrations of NA and 5 HT in the various parts of the rabbit spinal cord may be explained by similar differences in the amount of grey matter (calculated from the illustrations in Craigie 1949 p 77) In the cat the differences in concentrations of NA and 5 HT between the enlargements and the thoracic cord may be accounted for by a similar distribution of the grey matter (calculated from illustrations in Rexed 1954) However the cranial cervical segments of the cat cord have lower concentrations of both NA and 5 HT than the other parts even if allowance is taken for the somewhat smaller content of grey matter In the conus medullaris there is a very high concentration of 5 HT actually the highest found in the central nervous system of cats in this laboratory The hypothalamus which has the second highest 5 HT concentration contains about 0 7 μg/g The cellular localization of the 5 HT in the cat sacral region has been studied by the histochemical fluorescence method (Dahlstrom and Fuxe unpublished data) In the anterior horns there are abundant 5-HT terminals especially in the ventral and ventrolateral parts and around the central canal In the superficial zone of the posterior horns there are numerous descending 5-HT axons the terminals of which probably are submicroscopic Further there are many 5-HT terminals in the twisted parasympathetic nucleus (Rexed 1954) but not as many as in the sympathetic column of the thoracolumbar cord

From the results on the distribution of DOPA decarboxylase activity in the rabbit and cat spinal cord it may be concluded that the enzyme and the amines occur at the same sites like in the brain (Bertler and Rosengren 1959 a and b Kuntzman *et al* 1961) This view is in harmony with the observations that the DOPA decarboxylase like the amines is localized in descending nerves of the spinal cord (Andén Magnusson and Rosengren 1964 a and b) and that it probably catalyzes the biosynthesis of both catecholamines and 5 HT (Westermann *et al* 1958 Yuwiler *et al* 1959 Bertler and Rosengren 1959 b Rosengren 1960)

The results from the investigations of the distribution of amines and DOPA decarboxylase activity in the thoracic cord must be looked upon as preliminary especially with regard to the facts that the dissection was technically difficult and longlasting Further during the preparation there apparently was a considerable breakdown of the amines (see Table III *cf* Bertler and Rosengren 1959 a) There is however little doubt that both monoamines have their largest accumulation in the lateral horns and are almost

completely lacking in the white matter. These findings are in agreement with the observations made by the histochemical technique (Carlsson *et al.* 1964). Because of the small weight of the lateral horns their monoamines do not contribute by more than at most 10 per cent of the total monoamine content of the thoracic cord. This fact probably explains why the monoamine concentrations of the thoracic cord are not larger than those of the other cord parts in the rabbit and cat experiments. The discrepancy between the distribution of the amines and the DOPA decarboxylase activity in the cow thoracic cord as well as the high decarboxylase activity in the whole thoracic cord of this species are obscure. The histochemical method has shown that the monoaminergic nerves in the spinal cord begin to display varicosities with high amounts of monoamines prior to their entrance in the grey substance where all synaptic contact appears to take place (Carlsson *et al.* 1964). Part of the DOPA decarboxylase in the white matter may be localized in such varicosities. Also the non terminal parts, however, of the monoaminergic neurons contain this enzyme (Holtz and Westermann 1956, Andén, Magnusson and Rosengren 1964 b). It may be conceivable that the descending monoaminergic axons occupy a larger part of the white matter of the thoracic cord of cows than of the smaller animals. If the relatively low DOPA decarboxylase activity found in the lateral horns is not due to technical errors it may possibly indicate a lower synthesis rate in the nerves belonging to the central part of the autonomic nervous system than in those belonging to other systems. The proportion between DOPA decarboxylase activity and amine concentration in the lateral horns is similar to that found in sympathetically innervated organs (Andén, Magnusson and Rosengren 1964 b) but lower than that found in other parts of the central nervous system (Bertler and Rosengren 1959, unpublished data).

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From the Department of Physiological Chemistry University of Lund Sweden

The Metabolism of Fatty Acids in the Rat

VII Linoleic Acid

By

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Abstract

Goransson G *The metabolism of fatty acids in the rat VII Linoleic acid* Acta physiol scand 1965 64 204—710 — 1 C linoleic acid and H palmitic acid complexed with serum were simultaneously injected into normal rats of two nutritional states and the distribution of radioactivity investigated It was found that the labeled linoleic acid disappeared more rapidly from the blood than the labeled palmitic acid At 5 min less linoleic acid than palmitic acid label was *recovered from the fasted rats The distribution of radioactivity between the neutral lipid* and phospholipid fractions in the separate tissues from the fasted rats agreed with the fatty acid composition of each fraction The pattern of radioactivity in the blood phospholipids at 370 min closely resembled that in the liver phospholipids at 5 min

Previous investigations in our laboratory have been concerned with the metabolism in the rat of six different fatty acids (Goransson and Olivecrona 1964 and 1965 Goransson 1964 a b c d)

The present article reports the results from experiments with linoleic acid The purpose was to study if this essential fatty acid is metabolized differently compared to the non essential palmitic acid in normal rats The two acids labeled with C and H^3 respectively were therefore injected together into rats of different nutritional state and the following parameters studied disappearance of label from the blood uptake of label into the tissues oxidation of label incorporation of label into tissue lipids and recirculation of label back into the blood

Methods

The condition of the rats the operative procedures and the analyses performed on the samples have been described earlier (Goransson and Olivecrona 1964 and 1965) In the present work special care was taken to prevent autooxidation of the polyunsaturated fatty acid

The 1 C^{14}-cis-cis linoleic acid (Batch 20) was obtained from The Radiochemical Centre Amersham England Its specific activity was 247 mc/mM The radiochemical impurities

amounted to 10 and the chemical impurities as trans isomers to 9 and as conjugated diene to 4 / This purity was not considered sufficient for the purpose of injection into rats and comparison with palmitic acid containing less than 0 5 of impurities. Therefore the linoleic acid was first subjected to reversed phase chromatography with 75 ethanol/25 water as mobile phase and heptane as stationary phase 14 of radiochemical impurities were discarded After extraction from an acidified ethanol/water mixture the linoleic acid was methylated with diazo-methane The methyl esters were separated into the pure trans form the mixed cis trans form and the pure cis form by thin layer chromatography (Privett Blank and Romanus 1963) As reference substances were used cis-cis methyl linoleate (Fluka AG Basel Switzerland) and trans trans methyl linoleate prepared as described by McCutchon *et al* (1959) The methyl esters were eluted from the silica gel with ethyl-ether 84 of the radioactivity on the thin layer plate was recovered as pure cis methyl linoleate whereas 10 were trans-trans and cis trans-methyl linoleate. Furthermore 6 of unidentified impurities were found between the cis-cis-methyl linoleate and the origin

The methyl esters were hydrolyzed with 4 KOH in 95 ethyl alcohol in water at room temperature overnight The fatty acid was finally extracted with petroleum ether from an acidified alcohol/water mixture and kept in benzene under nitrogen atmosphere at —20 C

Analysis of a methylated sample of the cis-cis linoleic acid by thin layer chromatography showed that less than 0 5 of impurities were present.

The 9 10 H^3 palmitic acid used in the present work was also obtained from The Radiochemical Centre Amersham England It was purified by reversed phase and thin layer chromatography to assure that all the radioactivity was present as free palmitic acid

An injection solution of the two acids in rat serum was prepared as described earlier (Goransson and Olivecrona 1964 and 1965) The injected dose of 0 5 ml of serum contained approximately 0 03 micro-Eq of both the labeled linoleic acid and the labeled palmitic acid

Results

In the present experiments the behaviour of the labeled palmitic acid agreed with the results of earlier work (Goransson and Olivecrona 1964) Therefore in this article the the results are given as the ratio of linoleic acid label (C)/palmitic acid label (H^3) The ratio in the injected fatty acids was taken as one

The results can be seen in Tables I—IV

Table I shows the disappearance of label from the blood Linoleic acid was extracted from the circulating blood at a higher rate than palmitic acid in both fasted and refed rats The refed rats showed the greatest difference

Table II gives the ratios between C label and H^3 label in blood lipid fractions at 40 and 320 min These time intervals after injection were chosen because earlier work (Goransson and Olivecrona 1964 Laurell 1959) has shown that the recirculation of labeled fatty acid in the triglyceride fraction of blood or plasma reaches a maximum around 40 min Furthermore considerable amounts of label do not appear in the blood phospholipid and cholesterol ester fractions until several hours after the injection of the labeled fatty acids The present results indicate that at 320 min linoleic acid label had been incorporated to a larger extent into the blood cholesterol esters than palmitic acid label both in the fasted and the refed rats At 40 min the blood glycerides of the fasted rats contained less linoleic acid label than palmitic acid label whereas the reverse was true in the refed rats In the phospholipids from both fasted and refed rats the ratio of linoleic acid label to palmitic acid label was smaller than unity at 320 min

In Table III the radioactivity of the total lipid extracts from different tissues and from the whole rats can be seen The labeled linoleic acid was more rapidly oxidized than the palmitic acid label in the fasted rats but in the refed rats the ratio between linoleic and palmitic acid label was rather close to unity The same tendency was found in the separate tissues In the fasted rats the ratio was well below one in all the tissues

TABLE I C /H³ radioactivity in the blood FFA fraction in rats after the i v injection of C⁴ linoleic and H palmitic acid in rat serum Each value is the mean ± SEM of 3 rats The ratio in the injected fatty acids was taken as 1 0

Min	Fasted rats	Refed rats
1	0 68 ± 0 04	0 38 ± 0 02
2	0 50 ± 0 05	0 31 ± 0 04
3	0 47 ± 0	0 29 ± 0
4	0 43 ± 0 06	0 24 ± 0 04
5	0 44 ± 0 03	0 19 ± 0 03

TABLE II Ratio C /H radioactivity in the blood lipids in rats after the i v injection of C linoleic and H palmitic acid in rat serum Each value is the mean ± SEM of 3 rats The ratio in the injected fatty acids was taken as 1 0

Min	Fasted rats			Refed rats		
	Cholesterol esters	Glycerides	Phospholipids	Cholesterol esters	Glycerides	Phospholipids
40	0 86 ± 0 12	0 80 ± 0 09	0 73 ± 0	1 43 ± 0 20	1 13 ± 0 03	0 81 ± 0 04
320	1 97 ± 0 21	1 17 ± 0 08	0 69 ± 0 02	1 48 ± 0 13	1 05 ± 0 05	0 55 ± 0 09

TABLE III Ratio C H radioactivity in the total tissue lipids from rats after the i v injection The ratio in the injected fatty acids was taken as 1 0 Total in the rat represents

Min	Liver	Adipose tissue	Muscle	Heart
	Fasted rats			
5	0 84 ± 0 02	0 83 ± 0 02	0 89 ± 0	0 72 ± 0 03
320	0 69 ± 0 02	0 62 ± 0 22	1 00 ± 0 05	0 73 ± 0 01
	Refed rats			
5	0 97 ± 0 01	0 82 ± 0 01	0 85 ± 0 01	1 07 ± 0 02
320	0 87 ± 0 02	1 04 ± 0 03	0 87 ± 0 01	1.54 ± 0 15

TABLE IV Ratio C [14]/H rad oactivity in the neutral l p d and the phospholip d fractions of tissue lipids from fasted and refed rats after the i injection of C linoleic and H palmitic acid in rat serum. The values represent the mean ± SEM of 3 rats The ratio in the injected fatty acids was tak n as 1 0

Min		Liver	Muscle	Kidneys	Lungs	Spleen
	Fasted rats					
5	Neutral lipids +FFA	1.02 ± 0.03	0.83 ± 0.01	0.67 ± 0.07	0.54 ± 0.01	0.35 ± 0.01
	Phospholipids	0.71 ± 0.01	1.10 ± 0.06	0.58 ± 0.05	0.44 ± 0.01	0.62 ± 0.03
320	Neutral lipids +FFA	0.93 ± 0.04	0.99 ± 0.05	0.57 ± 0.01	1.21 ± 0.14	0.43 ± 0.09
	Phospholipids	0.62 ± 0.02	0.99 ± 0.08	0.60 ± 0.06	0.35 ± 0.04	0.46 ± 0.01
	Refed rats					
5	Neutral lipids +FFA	0.99 ± 0.02	0.54 ± 0.02	0.58 ± 0.01	0.52 ± 0.01	0.39 ± 0.02
	Phospholipids	1.06 ± 0.02	1.31 ± 0.06	1.03 ± 0.03	0.90 ± 0.03	0.82 ± 0.01
320	Neutral lipids +FFA	0.97 ± 0.01	0.56 ± 0.03	0.76 ± 0.02	0.81 ± 0.01	0.85 ± 0.14
	Phospholipids	0.87 ± 0.04	1.09 ± 0.01	0.91 ± 0.02	0.74 ± 0.02	0.65 ± 0.01

of C linoleic and H palmitic acid in rat serum Each value is the mean ± SEM of 3 rats the ratio in the sum of th rad oactivity in all the tissues taken out plus that in the carcass

Kidneys	Lungs	Spleen	Carcass	Total in the rat
0.59 ± 0.05	0.43 ± 0.01	0.51 ± 0.01	0.87 ± 0.01	0.84 ± 0.01
0.58 ± 0.03	0.36 ± 0.01	0.4[illegible] ± 0.01	0.95 ± 0.04	0.86 ± 0.03
0.89 ± 0.02	0.64 ± 0.01	0.65 ± 0.01	0.97 ± 0.04	0.95 ± 0.03
0.90 ± 0.0[illegible]	0.65 ± 0.02	0.60 ± 0.01	1.00 ± 0.01	0.97 ± 0.01

and in the refed rats the ratio was higher in all tissues than in the fasted rats In the livers from both fasted and refed rats the ratio at 320 min was smaller than that at 5 min

Table IV finally, shows the relative incorporation of linoleic acid label to that of palmitic acid label into the neutral fat + FFA and the phospholipids In some tissues from fasted rats more linoleic acid label than palmitic acid label was incorporated into the phospholipids as compared to the glycerides after 5 min and in other tissues the reverse was true In the refed rats however there was always more linoleic acid label in the phospholipids at 5 min than in the glycerides

Discussion

Earlier experiments by Mead *et al* (1956) and by Blomstrand (1954) showed that when fed in excess of amounts needed linoleate was oxidized more rapidly than fed stearate and palmitate in the rat Lossow and Chaikoff (1959) found that the oxidation of linoleic and linolenic acid was spared by glucose Thus in these investigations no specific sparing of the essential fatty acids was noted The same conclusion can be drawn from the present experiments in which the injected linoleic and palmitic acids were metabolized by the same principal pathways

Cis cis linoleic acid 1 C^{14} was found to disappear from the blood at a considerably higher rate than the H^3 palmitic acid both in fasted and refed rats In experiments carried out on humans Fredrickson and Gordon (1958) originally did not observe any difference in the extraction rate of i v injected linoleic and palmitic acid Later however Dustin *et al* (1961) injected H^3 palmitic acid and 1 C^{14} linoleic acid together into rats and noted that the C^{14} label disappeared more rapidly than the H^3 label from the blood These findings together with the results presented in this work make it probable that in the rat linoleic acid is in fact extracted at a higher rate from the blood than palmitic acid

At 5 min after injection practically all the labeled linoleic and palmitic acid had disappeared from the blood in the refed rats Therefore the total tissue radioactivity in the refed rats at 5 min after the injection most likely reflects the initial distribution of label since at that time little oxidation or redistribution of label between separate organs has taken place (Goransson and Olivecrona 1964) A ratio of linoleic acid label to palmitic acid label greater than unity was only found in the heart This might not have been expected in view of the results of Carlsten *et al* (1963) who showed that the a v difference over the human heart for linoleic acid was smaller than that of all other fatty acids studied In the lungs and the spleen the ratio C^{14}/H^3 suggested a preferential uptake of palmitic acid

Once the labeled fatty acids have been taken up by the cells they are directly available for oxidation interconversion and incorporation into separate lipid fractions followed by recirculation back into the blood as fatty acid esters The different pathways will be discussed in some detail below

Oxidation

In the fasted rats less linoleic acid label than palmitic acid label was recovered from the whole rat 5 min after injection It should be recalled at this point however that the linoleic acid label disappeared more rapidly from the blood than the palmitic acid

label Thus linoleic acid and palmitic acid may still be equally available for oxidation immediately after the entry into the cell in spite of the low ratio obtained in the present experiments After 320 min the amount of the two isotopes had declined parallelly so that the ratio was still the same This is partly explained by the fairly equal incorporation of the two isotopes into neutral lipids and phospholipids

The finding in this work that linoleic acid was more rapidly oxidized than palmitic acid agrees with earlier results (Mead *et al* 1956 and Blomstrand 1954) that indicated a more rapid oxidation of fed linoleic acid than palmitic acid in rats

Inte conversion

Theoretically linoleic acid might be oxidized to 2-carbon units which could then give rise to fatty acids other than linoleic In the present experiments however this indirect interconversion does probably not take place to any large extent because in the refed rats the overall oxidation is very small and in the fasted rats the synthesis of fatty acids from 2-carbon units is depressed

Another way of interconversion would be hydrogenation, but this reaction is considered to be of minor importance in higher animals (Mead 1960)

The conversion of linoleic acid to arachidonic acid constitutes a third possible way of interconversion That this reaction does indeed take place was shown by Mead (1953) However in the experiments of Dittmer and Hanahan (1959) labeled arachidonic acid was not found to any large extent except in phosphatidyl-ethanolamine (26 %)

The interconversions of palmitic acid have been discussed elsewhere (Elovson 1965)

Incorporat on of radioactivity into neut al lipids and phospholipids

In the fasted rats where no interconversion of palmitic acid takes place it was found that incorporation of linoleic acid label as compared with palmitic acid label took place according to the fatty acid composition in the respective lipid fraction found earlier (Goransson and Olivecrona 1964) except in the spleen

Recir ulat on back into the blood

As could be expected from earlier work (Goransson 1964 a and b the pattern of label in the blood phospholipids at 320 min was similar to that in the liver phospholipids at 5 min The ratio in the blood glycerides did not resemble the ratio in the liver neutral lipid f action as closely as in earlier work with other fatty acids

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Direct Studies on the Disappearance of the Transmitter and Changes in the Uptake-Storage Mechanisms of Degenerating Adrenergic Nerves

By

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Abstract

Malmfors T and Ch Sachs *Direct studies on the disappearance of the transmitter and changes in the uptake storage mechanisms of degenerating adrenergic nerves* Acta physiol scand 1965 64 211—223 — The histochemical fluorescence method of Falck and Hillarp has been applied to a study of the disappearance of the endogenous noradrenaline (NA) and the uptake storage mechanisms of the adrenergic nerves in the rat iris during the course of degeneration following axotomy It was found that the transmitter stored in the terminals does not disappear gradually but quite suddenly and with various times of onset for the different systems of terminals The uptake and accumulation of NA and α methyl NA were examined in untreated and reserpinized animals. These experiments show that the uptake-storage mechanisms in any given system of terminals operate up to a certain time without any obvious changes but then deteriorate rapidly at about the same time as the transmitter stores disappear The axon membrane reabsorption mechanism seems to be lost somewhat prior to the storage mechanism of the transmitter granules at the same time as degenerative changes seem to occur in the axon membrane making possible a passive leakage of NA — The results add further weight to the view that the cocaine like component in the denervation supersensitivity is due to the loss of the uptake mechanisms — Following the administration of bretylium the degenerative processes were delayed for about 8 hrs

The disappearance of the transmitter and the degenerative changes occurring in the adrenergic nerves following axotomy have recently been studied with renewed interest (see *e g* Kirpekar *et al* 1962 Trendelenburg 1963 a and b Benmiloud and Euler 1963 Fleming 1963 These studies have concerned themselves especially with current hypotheses as to the cause of denervation supersensitivity and it has been proposed that loss of the mechanisms for amine uptake is to a great extent responsible for such supersensitivity as it permits higher concentrations of catecholamines (CA) to reach the adrenergic receptors

The adrenergic nerves and their transmitter can now be studied at cellular and subcellular levels in an entirely new and direct manner using the highly sensitive fluorescence method of Falck and Hillarp for the histochemical demonstration of certain monoamines As shown previously (Malmfors 1965 a) this can be done especially successfully on whole mounts of albino rat iris which permit a close examination of the adrenergic axons and terminals throughout the tissue with respect to the occurrence distribution uptake and storage of the transmitter

In the present work, the rat iris technique has been used for studies of the changes occurring in the nerves during the course of degeneration following axotomy with respect to the intraneuronal distribution and disappearance of the transmitter the loss of amine uptake-storage mechanisms and the effects of reserpine bretylium and *a potent monoamine oxidase (MAO) inhibitor (nialamide)*

Material and methods

Changes in the adrenergic nerves were studied for the most part on whole mounts of rat iris during the *course of degeneration following axotomy Investigation covered 1) the intraneuronal* distribution of the transmitter 2) its disappearance and 3) the uptake accumulation and disappearance of CA following the administration of dopamine (DA) noradrenaline (NA) and α methyl NA (and also L-3 4-dihydroxyphenylalanine L-DOPA) The study is based on the principle that the transmitter can be directly demonstrated by means of a histochemical fluorescence reaction (Falck *et al* 1962)

The irises of about 300 adult female albino rats Sprague Dawley weighing about 200 g were used Cervical sympathectomy was performed by bilateral extirpation of the superior cervical ganglion together with 5 to 6 mm of the preganglionic trunk and 1 to 2 mm of the postganglionic nerves under ether anesthesia 2 4 8 12 16 20 28 32 36 and 40 hrs before death Most of the animals were treated with one or several of the following drugs reserpine (Serpasil Ciba) nialamide (Niamid Pfizer) NA α methyl NA (Corbasil Hoechst) DA L-DOPA and bretylium tosylate All drugs were given intraperitoneally except bretylium tosylate which was given s c The animals were killed by decapitation under light ether anesthesia

The eyes were removed immediately and the irises prepared as whole mounts which after drying were treated with formaldehyde gas of optimum humidity (see Hamberger *et al* 1965) at plus 80 C for 1 hr The techniques used for preparing the irises and for their examination in the fluorescence microscope are described in detail in a previous paper (Malmfors 1965 a) To check the observations made on the whole mounts one of the irises was on several occasions freeze-dried and — after formaldehyde treatment — embedded in paraffin and *sectioned in 10 μ thick sections which were mounted in Entellan (Merck for details see* Dahlström and Fuxe 1964) The same results were obtained in both the freeze-dried irises and the air-dried whole mounts

Most of the irises were examined once or twice without our knowing the pretreatment given to the animal The strongly fluorescent terminals of normal appearance remaining at the different times of degeneration were estimated roughly by a careful examination of the entire iris No great differences were found between the two observers

Results

1 Changes in content and distribution of the adrenergic transmitter during the course of degeneration following axotomy

The histochemical treatment results in a conversion of NA (and also of DA DOPA and their α methylated derivatives into 3 4-dihydro isoquinolines which exhibit an intense fluorescence (green to yellow-green in the fluorescence microscope Falck *et al* 1962 Corrodi and Hillarp 1963 1964) Thanks to the extremely high sensitivity of the method (see Norberg and Hamberger 1964) the adrenergic transmitter is readily demonstrated and its intraneuronal distribution and the adrenergic innervation apparatus can thus be studied

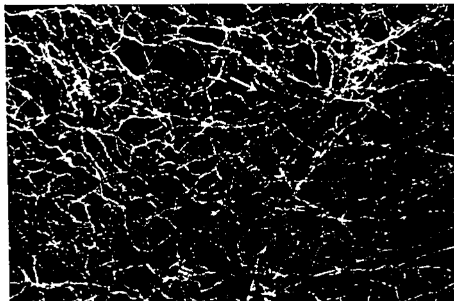
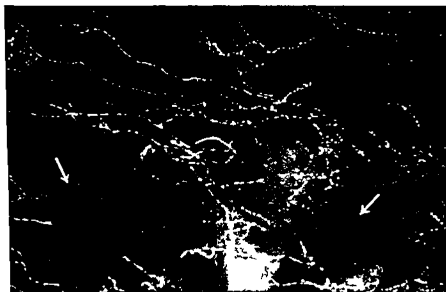


Fig 1 Iris of normal rat In addit on to the strongly fluorescent and un formly distributed plexus of adrenerg c n rve terminals over the d lator muscle and a ound an arteriole there is seen a nerve trunk with weakly fluorescent non varicose main axons (⟶) × 160



I ⁊ Rat r 16 l fter axotomy Th ma n p t f tl e nerve plexus has d sappea l l av ng son bran h ter inals of normal appearance (type N) No f bres of type D ar p esent Some preterminal fibres are seen (—) × 160

Fig 3 Rat iris 16 hrs after axotomy. Animal treated with nialamide 4 hrs and noradrenaline 1 hr before preparation. The non terminal axons have a greatly increased fluorescence intensity (⟶) as have the varicosities of the D type terminals. Type N terminals are seen forming a continuous system in the lower middle of the picture. × 160

Fig 4 Rat iris 16 hrs after axotomy. Animal treated with nialamide 4 hrs before preparation. In the middle of the picture are seen one or two systems of strongly fluorescent terminals type N extended very like a system of terminals after partial denervation of the iris (Malmfors and Sachs 1965). Type D terminals can be observed with more strongly fluorescent varicosities than in operated animals not pretreated with nialamide. Non terminal axons are also seen

The adrenergic innervation of the iris has been described in detail in other papers (Malmfors 1965 a and b Malmfors and Sachs 1965) The sympathetic fibres arrive at the periphery of the iris These main axons continue in small bundles (Fig 1) and finally give off several thin smooth branches preterminal axons (Fig 2) Each of the preterminal axons ends with a system of branching terminals (Fig 4) which have abundant enlargements (varicosities) The varicosities — in contrast to the thin fibre segments between them — exhibit a strong fluorescence due to the presence of very high concentrations of NA There is little doubt that practically all this amine is stored in the varicosities and that these are specialized structures for the synthesis, storage and release of the transmitter (Norberg and Hamberger 1964 Malmfors 1964 ... The non terminal axons have low transmitter concentrations and show at the most weak fluorescence — The innervation apparatus in normal animals appears as a dense 2-dimensional net work each strand of which usually contains 2 or more terminals This is due to the facts that the terminals run ensheathed in Schwann cells forming the anastomosing strands of the autonomic ground plexus (see Hillarp 1959) and that two or more systems of terminals often converge to the same effector cells — Further microphotos of normal and degenerating iris nerves are to be found in Malmfors (1965a)

Irises from about 70 animals were examined from 8 to 40 hrs after axotomy No obvious changes in the appearance of the adrenergic nerves were found at 8 hrs After 48 hrs or more the stored transmitter had either completely disappeared or else — in about 10 per cent of cases — there were left a few systems of branching and apparently intact terminals no doubt originating from 1 or 2 sympathetic ganglion cells located peripherally to the axotomy (see Malmfors and Sachs 1965)

A closer examination of the degeneration changes revealed the following

a) The transmitter stored in the terminals did not disappear gradually from the entire innervation apparatus at once System after system of branching terminals distributed more or less randomly throughout the iris (Fig 2) lost their NA content completely while — at any given point of time after axotomy — most of the remaining terminals appeared normal (type N) and the rest had only weakly fluorescent varicosities and exhibited no only or a very weak fluorescence in the segments between these (type D) Rough estimates of the numbers of these two types of terminals remaining at different degeneration times are found in Table 1 The figures for 12 hrs are probably too high since it is difficult to estimate the magnitude of a disappearance when most of the terminals still remain There is little doubt however that a large proportion of the terminals were affected and lost their NA during the four hours between 12 and 16 hrs after axotomy This and the finding that no clear transitional stages seemed to exist between the type N and type D terminals show that the terminals at some point of time — which differs from system to system — lose their transmitter content quite suddenly (probably within 1 or at the most 2 hrs) The same findings also seem to indicate that degeneration proceeds in 2 phases first a rapid disappearance of most of the stored amine and then a somewhat slower disappearance of the remaining contents

The branching terminals belonging to the system arising from a single preterminal fibre reacted uniformly along their entire lengths during the course of degeneration This could be observed especially at 16 and 20 hrs after axotomy when many or most of the terminals had disappeared completely The remaining systems of terminals of both types were distributed more or less randomly throughout the iris The same findings were made in the experiments with nialamide treatment and amine uptake described below

The main postganglionic axons and the preterminal fibres seemed to lose their low NA content at approximately the same time after axotomy as the terminals Numer

ous non terminal axons were seen and their course and branching could be easily tudied at 16 and 20 hrs (Fig 2 3) None or very few remained at 24 hrs. The earliest sign of degeneration was the appearance of usually small enlargements irregularly distributed along the fibres

Differences in the time course of the degeneration were observed between the two irises from the same animal but were greater from animal to animal at the same degeneration time

2 *Reserpine induced depletion of the adrenergic transmitter during degeneration*

A large dose of reserpine (10 mg/kg) was administered to 3 groups of 4 rats immediately after cervical sympathectomy and the animals examined after 2 4 and 8 hrs Just as in animals with intact innervation (see Malmfors 1965 a) the fluorescence was strongly reduced after 2 hrs while very few and weakly fluorescent terminals remained at 4 hrs and all fluorescent nerves had disappeared after 8 hrs

3 *Effects of nialamide treatment during degeneration*

The adrenergic nerves were examined 4 hrs after a single large dose of nialamide (500 mg/kg 42 rats degeneration times from 12 to 36 hrs) or 4 hrs after the last of 2 to 6 doses 100 mg/kg 18 rats, degeneration times (from 16 to 24 hrs) administered at intervals of 4 hrs

The administration of this potent MAO inhibitor did not result in any obvious delay in the disappearance of the transmitter (Table 1) The terminals of type N remaining at the various times showed a normal appearance but a distinctly increased fluorescence intensity A generally increased transmitter content has also been found in the terminals of animals with intact innervation Malmfors 1965 a No type D terminals with weakly fluorescent varicosities were observed but there were found instead terminals similar to type D terminals in respect of their occurrence at 16 and 20 hrs and the weak to non-existent fluorescence of the segments between the varicosities The latter however were more pronounced and exhibited a strong fluorescence (Fig 4 There seems to be little doubt that these terminals are identical with the type D terminals see Discussion — The non terminal axons showed an increased fluorescence intensity (Fig 4

4 *Amine uptake mechanisms during degeneration*

It has been shown in previous papers (Hamberger et al 1964 Hillarp and Malmfors 1964 Malmfors 1965 a that the entire postganglionic axon possesses a mechanism for the uptake-concentration of NA and related amines which is localized to the level of the axon membrane This mechanism can be studied after the administration of a large dose of reserpine which causes a complete depletion of the endogenous NA and blocks the storage function of the granules (Carlsson 1965)

It is possible directly to demonstrate the uptake of NA or α methyl NA following the administration of very low doses of the amines i v or intraocularly In the present experiments however it was found valuable to give high doses 1 and sometimes up to 10 mg/kg i p in order to obtain a very marked accumulation of the amines 1 hr after their administration In most of the experiments 46 rats α methyl NA was given alon and NA 4 to 5 hrs after the administration of nialamide 100 mg/kg since NA — but not α methyl NA — breaks down very rapidly intraaxonally if the granul mechanism is not intact or MAO not efficiently inhibited cf Malmfors 1965a

TABLE I Rough estimates per cent of remaining adrenergic terminals of normal appearance (N) or with weak fluorescence (D)

Degeneration time Hrs	Untreated	Nialamide	Nialamide + NA (or α-methyl NA)	Reserpine + nialamide + NA (or α-methyl NA)	Bretylium tosylate
	Treatment of the animals				
8	100 (N)			100 (N)	
12	97—100 (N)	95—100 (N)	95—100 (N)	95—100 (N)	
16	25— 40 (N)	25— 50 (N)	25— 45 (N)	30— 50 (N)	
	10— 20 (D)	10— 15 (D)	10— 20 (D)		
20	5— 15 (N)	10— 15 (N)	10— 20 (N)	5— 10 (N)	90— 95 (N)
	2— 5 (D)	1— 5 (D)	1— 5 (D)		
24	0— 2 (N)	1— 3 (N)	1— 4 (N)	0— 1 (N)	25— 45 (N)
					5— 10 (D)
28	0— 1 (N)	0— 1 (N)	0— 1 (N)	0	

Disappearance of the transmitter in the adrenergic terminals of the rat iris after cervical sympathectomy —The rats — usually 5 to 7 animals in each group — were either untreated or treated with one or several doses of nialamide or bretylium tosylate The amine uptake mechanisms of the degenerating terminals were studied in rats killed 1 hr after a large dose of NA (pretreatment with nialamide) or α methyl NA. In one of these experiments the endogenous NA was first depleted by administration of a large dose of reserpine 1 hr after the operation — The terminals of type D were degenerating and had lost most of their transmitter (weakly fluorescent varicosities) After nialamide treatment or the administration of NA however the corresponding terminals (marked with an asterisk) showed strongly fluorescent varicosities (see Sections 3 and 4)

The same results were obtained in both cases The effects of degeneration on uptake were studied at 12 to 40 hrs after axotomy but most of the experiments were made at the critical times of 16 and 20 hrs

No accumulation could be obtained after degeneration times of 24 hrs or longer Large doses of DA (5 to 100 mg/kg i p) or L-DOPA (20 to 100 mg/kg i p) to animals (30) pretreated with nialamide were also entirely ineffective

The amines accumulated in the adrenergic nerves in normal or reserpinized animals remain for many hours provided that MAO activity is efficiently inhibited (see Malmfors 1965 a) However no obvious delay was observed (Table I) in the disappearance of the two types of terminals in spite of the fact that high accumulations similar to those obtained in intact nerves found in the degenerating nerves at 12 hrs — when all the terminals seemed to remain — and in the terminals remaining at 16 and 20 hrs The type N terminals exhibited a very strong fluorescence and the increase — just as in normal terminals — was most marked in the segments between the varicosities which gave the fibres a more smooth appearance No weakly fluorescent type D terminals

could be observed but terminals which no doubt correspond to this type had varicosities exhibiting a very strong fluorescence and seemed also to be larger (this may be a degenerative swelling which can be clearly seen only after a high accumulation of amines) In marked contrast to intact terminals no or only a very weak fluorescence could be obtained in the segments between the varicosities (Fig 3) — No increase in fluorescence was observed 30 min after the administration of NA alone

Many non terminal axons showed a high amine accumulation 16 and 20 hrs after axotomy their course and branching could be readily followed in the entire iris and their rate of disappearance could also be successfully studied (Fig 3) In agreement with the findings reported in the previous section the fluorescence of these axons seemed even after a massive accumulation of amines to disappear at about the same time as the terminals — or perhaps somewhat later — since many strongly fluorescent non terminal axons were observed 20 hrs after axotomy

5 *Amine uptake mechanisms during degeneration in reserpinized animals*

The uptake and accumulation of NA or α methyl NA (for doses and times see section 4) were examined in 70 rats given reserpine (10 mg/kg) 1 hr and killed 8 to 32 hrs after axotomy Unless otherwise stated nialamide was administered 4 hrs before NA About the same results were obtained with nialamide plus NA as with α methyl NA alone

No accumulation was found at degeneration times of 24 hrs or longer Terminals showing a high amine accumulation were found at 8 to 20 hrs after axotomy with a frequency and distribution about the same as those of the type N terminals in untreated animals (Table I) These terminals — just as in reserpinized animals with intact innervation — showed a more smooth appearance with a strong fluorescence also in the segments between the varicosities No terminals were observed however that would correspond to type D with strongly fluorescent varicosities but exhibiting no or only weak fluorescence in the segments between

No NA was found to remain 30 min after administration in animals not pretreated with nialamide unless the rats were killed within 15 min after the NA injection and the irises left *in situ* for 15 min before being taken out This treatment produces anoxia in the iris and thus an inhibition of MAO (see Malmfors 1965 a)

The non terminal axons showed a high amine accumulation and many remained at 20 hrs after axotomy just as in the non reserpinized animals in the previous series

The disappearance of the NA accumulated 4 to 8 hrs before killing of the animals was examined in 16 rats 12 to 20 hrs after axotomy The strongly fluorescent terminals disappeared at about the same rate as type N in untreated animals The terminals remaining at 12 and 16 hrs showed a reduction in their accumulated amines that was similar to the terminals in reserpinized animals with intact innervation (see Malmfors 1965 a)

6 *Effects of bretylium during degeneration*

Bretylium tosylate was administered in doses of 50 mg/kg at intervals of 4 hrs starting immediately or 8 hrs after cervical sympathectomy The animals (12) were killed 20 or 24 hrs after operation

In contrast to the untreated animals the rats treated with bretylium showed no or only a small decrease in the number of terminals or in their fluorescence intensity 20 hrs

after operation (Table I) Four hours later there were still many strongly fluorescent terminals of apparently normal appearance and there were observed now also type D terminals As seen in Table I however the disappearance of the transmitter in the interval between 20 and 24 hrs was about the same as that in the interval between 12 and 16 hrs in the animals not treated with bretylium

The non terminal axons showed about the same fluorescence as in untreated animals They were numerous at 24 hrs when there was a marked reduction of the terminals

Discussion

The time course of the disappearance of the adrenergic transmitter in the brown adipose tissue (Weiner *et al* 1962) and the submaxillary gland (Benmiloud and Euler 1963) of the rat has been examined during degeneration of the sympathetic nerves The NA content was unchanged at 8 hrs but lost by 24 hrs after axotomy The present results are in complete agreement with these observations Our data on the disappearance of the adrenergic terminals (Table I) which contain practically the entire transmitter store agree well with the chemical data on the NA decrease in the submaxillary gland in the interval between 8 and 24 hrs since — as already pointed out — our 12 hr figures are in all probability too high —The adrenergic nerves in the rabbit iris have also been found to lose their NA gradually in the interval between 8 and 24 hrs after axotomy (Andén *et al* 1965) In other species degeneration seems to be somewhat slower (see Hillarp 1960 Kirpekar *et al* 1962 Wegmann *et al* 1962 Sedvall 1964)

Studies by the methods used hitherto on the disappearance of the transmitter during degeneration of the nerves have given little information as to the mechanisms of this disappearance and their relation to physiological or degenerative processes in the axons Although this has not been directly stated the results obtained seem generally to have been interpreted on the basis of the reasonable assumption that the rate of disappearance determined reflects by and large the events in the individual adrenergic nerves When the present study was started there was no obvious reason to doubt the validity of this assumption It shows clearly however that the transmitter does not disappear gradually over a long period from most or even from the greater part of the terminals as might have been thought All the observations show instead that any one of the systems of terminals — each consisting of the branching terminals arising from a single pre terminal axon — lose their NA content quite suddenly but that there are great differences between the systems in the time of onset The gradual decrease in tissue NA is consequently due to system after system of terminals dropping out more or less randomly throughout the tissue while the remaining systems mostly seem to be intact with respect to transmitter content but become fewer and fewer Although the terminals studied all belong both anatomically and functionally to the same population the numerous systems within the population show internally a great biological variation

The terminals remaining at any given time after axotomy did not for the most part (type N) differ from normal terminals with respect to morphology distribution of stored transmitter amine increase after administration of nialamide (*cf* Kopin 1964) or amine uptake and accumulation before or after reserpine (see below) Some of the terminals (type D) showed marked and characteristic changes The abundant va

ricosities that in normal terminals contain practically the entire amount of the NA stored in the adrenergic nerves and which are in all probability specialized structures for the storage and release of the transmitter (see Norberg and Hamberger 1964 Malmfors 1964 1965 a) had lost most of their amine content but increased in size which suggests that degenerative swelling had occurred As discussed below there is good evidence that these terminals had also lost one of the mechanisms for amine uptake and concentration Since there is little doubt that the terminals finally lo e the remaining transmitter and their property to accumulate amines at about the same time these findings indicate that the terminals belonging to one system are fairly normal up to a given time after axotomy but then lose most of their transmitter content quite suddenly probably due to degenerative changes which result in swelling and a deter ioration of the uptake-concentration mechanism localized to the level of the axon membrane After this rapid phase the remaining transmitter is lost somewhat more slowly probably due to deterioration of the uptake storage mechanism of the amine granules (see below) The degeneration thus seems to proceed in two phases

There is strong evidence that the storage granules in the adrenergic nerves isolated and extensively studied by Euler and co-workers (Euler and Hillarp 1956 Euler 1958 Euler and Lishajko 1961 1963 Stjarne 1964) contain most of the transmitter normally present in the non terminal axons and terminals and that the amines in this way are protected from inactivation by the MAO existing within the axons themselves (Hamberger *et al* 1964 Hillarp and Malmfors 1964 Malmfors 1965 a) It could further be directly demonstrated that not only the terminals but the entire postgang lionic axon possess a highly efficient mechanism for the uptake concentration of NA which is localized to — or at least to the level of — the axon membrane and — in contrast to the granules — is highly insensitive to reserpine There is little doubt that the mechanism for amine uptake by the granules is effectively blocked by reserpine (see also Carlsson 1965 Dahlstrom *et al*)

The observations on the uptake and accumulation of NA (or α methyl NA) by the terminals and non terminal axons clearly show that there is a very intimate correlation between the disappearance of the endogenous NA and severe changes in the uptake storage mechanisms The amines administered were readily taken up and accumulated to very high concentrations in the terminals both before and after a reserpine induced transmitter depletion up to 8 hrs after axotomy In contrast to this no accumulation at all could be obtained in any part of the axons at degeneration times of 24 hrs or longer The observations made on closer examination of the uptake storage mechanisms in the non reserpinized animals strongly support the view that these mechanisms in any given system of terminals operated up to a certain time without any obvious changes but then deteriorated rapidly and at about the same time as the transmitter stores disappeared This view received further strong support from the results obtained when the accumulation and disappearance of administered amines were studied during de generation in animals pretreated with a large dose of reserpine Of special significance is the finding that terminals showing about the same frequency and distribution at the various degeneration times as the type N terminals in untreated animals showed an efficient uptake and a very high capacity to concentrate the amines

No terminals of the D type were seen in the three groups of drug treated animals After nialamide treatment and even more pronouncedly after the administration of NA (nialamide pretreatment) or α methyl NA there appeared however terminals showing about the same distribution and frequency as the D terminals but which had

pronounced varicosities exhibiting a strong fluorescence These terminals — in contrast to the N terminals — showed no or only weak fluorescence in the thin segments of fibre between the varicosities There is thus little doubt that they are identical with the D terminals Terminals with normal axon membrane uptake-concentration mechanism take up and accumulate amines to very high concentrations both in the varicosities where the amine granules are highly concentrated (*cf* Norberg and Hamberger 1964) and in the segments between The fact that the D terminals showed an amine accumulation only in the varicosities is strong evidence that this mechanism had been lost but that the granules could still take up amines The results obtained in the experiments with amine uptake in the reserpinized animals strongly support this view No amine accumulation occurred in terminals of the D type but was observed in those of N type Since reserpine blocks the storage function of the granules but not the axon membrane mechanism this furnishes independent evidence that the degenerating terminals first lose the latter mechanism and then shortly afterwards the granule uptake mechanism

The administered amines accumulating in the intact adrenergic nerves of normal or reserpinized animals remain for many hours provided that MAO activity is efficiently inhibited (Malmfors 1965 a) Both accumulated and endogenous amines disappeared quite suddenly after axotomy however in spite of a highly efficient MAO inhibition This shows that the disappearance is not due to any intraaxonal degradation by the enzyme The results indicate instead that the transmitter is released from the terminals as proposed by Benmiloud and Euler (1963) on the basis of quite different observations This view is strongly supported also by the finding that there occurs a distinct mydriasis during the disappearance of NA from the adrenergic nerves in rabbit iris after axotomy (Andén *et al* 1965) This release could be thought to be a consequence of the loss of the uptake storage mechanisms There is good evidence however that the membrane surrounding the axons of intact adrenergic nerves effectively prevents the transmitter from leaking out even if the reabsorption mechanism of this membrane is strongly inhibited (Malmfors 1965 a) The rapid transmitter release is thus in all probability due mainly to a quite sudden change in the axon membrane which makes possible a passive leakage This change must occur at about the same time as when the axon membrane reabsorption mechanism deteriorates and it would seem to be responsible for the initial disappearance when most of the transmitter is rapidly lost An interesting possibility is that the degenerative processes in the axon membrane give permeability changes leading to *e g* a quite different ionic environment for the amine granules which thereby rapidly lose their capacity to retain their stored amines There is good evidence that amine storage granules are highly sensitive in this respect (Euler 1958 Hillarp 1958 Euler and Lishajko 1961 1963 and Carlsson *et al* 1963)

Benmiloud and Euler (1963) studied the effects of bretylium on transmitter depletion following axotomy and made the interesting observation that this drug may reduce or even prevent such depletion It was suggested that bretylium acts by blocking the normal release mechanism This prompted a direct study on the effects of this drug on the adrenergic terminals and their degeneration The finding made by Benmiloud and Euler was fully confirmed Unexpectedly however it was found that the degenerative processes in the terminals which lead to loss of the uptake mechanisms and the stored transmitter occurred just as in untreated animals with the only clear difference being that their time course as a whole was postponed *i e* the onset of the rapid phases

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On the Mechanism for the Stimulating Action of Reserpine on Acid Secretion in Gastric Fistula Cats

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Abstract

EMÅS S *On the mechanism for the stimulating action of reserpine on acid secretion in gastric fistula cats* Acta physiol scand 1965 *64* 224—237 — A single intravenous infusion of reserpine in sufficient to reduce antral gastrin activity significantly elicited acid secretion in nonanesthetized cats with innervated and sympathetically denervated stomachs Atropine or vagotomy reduced the acid response by 55 to 84 per cent In vagotomized cats atropine produced a smaller but significant reduction It was concluded that the vagal and sympathetic nerves are not necessary for reserpine to elicit acid secretion but that intact vagi and — to a lesser extent — the post ganglionic cholinergic neurons of the vagotomized cats enhance the effect of reserpine Reserpine treatment has previously been shown to sensitize the parietal cells to gastrin and to cause a transient reduction in antral gastrin activity in nonvagotomized but not in vagotomized cats In the present study reserpine treatment reduced the secretory response to intravenous reserpine in nonvagotomized cats and abolished the response in vagotomized cats A single intravenous infusion of reserpine elicited acid secretion in anesthetized cats after vagotomy and resection of the regions known or suspected to contain gastrin The results suggest a secretagogue action of reserpine which is not mediated by the vagi and by gastrin

The first papers reporting that a single injection of reserpine stimulated gastric acid secretion (Barrett Rutledge and Rogie 1954 Plummer *et al* 1954) have since been widely confirmed but the mechanism for the stimulating action has not yet been clarified

According to preliminary communications reserpine elicits acid secretion in the sympathetically denervated stomach (Emås 1961 Schapiro and Woodward *1961*) and experimental data in support in this are offered in the present paper It has also been shown that reserpine produces acid secretion after vagal denervation (Kirsner and Ford 1957 Rider Moeller and Gibbs 1957 Schneider and Clark 1957 Schapiro and Woodward 1961 Castiau and Reuse 1962 Kim and Shore 1963) but further studies are needed to evaluate the significance of the vagal nerves for the secretory effect of reserpine

The injection of 0 10 mg of reserpine per kg caused a release of gastrin from the antral mucosa of cats within 12 hrs The gastrin released was calculated to be sufficient

to maintain the gastric secretion of acid for more than 12 hrs The same dose did not, on the other hand affect antral gastrin activity after vagotomy A small dose of reserpine (0 030 mg per kg) produced no change of activity in nonvagotomized cats (Emås and Fyro 1965) If a single infusion of 0 030 mg of reserpine per kg or less elicits acid secretion in vagotomized cats this would accordingly indicate a stimulatory effect of reserpine independently of the vagi and the antral gastrin A reduced secretory response to reserpine was found to persist after vagotomy in the present study Since it has been suggested that reserpine evokes secretion by stimulating the parasympathetic ganglia (Barrett *et al* 1955 Kim and Shore 1963) the effect of atropine on reserpine induced secretion was investigated in vagotomized cats

The treatment with reserpine of nonanesthetized cats (0 10 mg per kg i m. daily) has previously been shown to sensitize the HCl secreting cells to infused gastrin (Emås 1963 1964 a) and to reduce antral and duodenal gastrin activity (Emås and Fyrö 1965) Vagotomy abolished these effects (Emås 1964 b Emås and Fyro 1965) By using nonvagotomized and vagotomized cats it was then possible to study the influence of reserpine treatment with and without its sensitizing and gastrin releasing effects on the acid response to a single infusion of reserpine Further evidence was obtained for a stimulatory effect of reserpine on acid secretion independently of the vagal nerves and gastrin

Methods

Operative Procedures

Experiments were performed on 15 nonanesthetized gastric fistula cats (Emås 1960) of both sexes (2 2 to 3 8 kg) In 3 animals (no 39 71 and 83) the distal parts of the thoracic sympathetic chains were resected (preganglionic sympathectomy) and in cats 71 and 89 the celiac ganglia were also removed (postganglionic sympathectomy) Bilateral thoracic vagotomy was performed on 9 cats one (no 71) of which had been pre and postganglionically sympathectomized respectively 22 and 14 months earlier Descriptions of the operations and of postoperative management have been given in previous papers (Emås 1964 a b)

The completeness of the vagotomy was tested by regular insulin in at least 2 expts on each animal as previously described (Emås 1964 b) After the reoperation of ? animals vagotomy was considered complete in all 9 cats The last test with insulin was carried out 3 to 12 months after vagotomy in 4 animals with no signs of vagal reinnervation. In the remaining cats the interval was shorter

Hair accumulated in the stomach in 2 of the 9 vagotomized cats in one cat (no 80 5 months in the other (no 94) 1 months after vagotomy The bolus of hair was removed through a gastrotomy and a pyloroplasty was performed as described in an earlier paper (Emås 1964 b

Anesthetized (chloralose urethane) gastric fistula cats (male and female 2.8 to 4 3 kg) were used in 7 expts The gastric fistula preparation was performed as previously described (Emås 1960) The vagal and sympathetic trunks were cut in the neck and the distal part of the stomach was resected from a level oral to the pyloric sphincter by 6 cm on the lesser and 7 cm on the greater curvature The anatomical antrum-corpus border was situated in the resected segment Since gastrin like activity has been demonstrated in the proximal duodenum of cats (Emås and Fyro 1965) and has been claimed to be present in the atrophic pancreas of dogs (Elliott *et al* 1963) 16 cm of the proximal duodenum and the pancreas was resected after ligation of the duodenum and the common bile duct The presence of a small gastrin like activity in the cardiac mucosa of hogs (Gregory and Tracy 1961) was not confirmed in cats (Emås and Fyro unpublished

Experimental Technique

At least 2 weeks were allowed after operation before the secretory studies were started on the nonanesthetized cats Experiments on the anesthetized cats were started 1— hrs after completion of the operation and the rectal temperature was maintained between 37 and 38 C throughout each experiment by means of an infrared lamp The animals were deprived of food and liquid for 18 to 24 hrs before each experiment.

The volume of gastric juice was recorded for 15 min periods and the amount of free and total acid determined by titration against 0 01 N NaOH with Topfer s reagent and phenol phthalein as indicators The amount of acid secreted was expressed in meq

After the basal output of acid had been determined for at least 1 hr reserpine (Serpedin®) freshly diluted in physiological saline (0 9 per cent NaCl solution) was infused i v for 15 min by means of a motor-driven infusion pump that delivered 0 40 ml/min The acid secretion induced by a single reserpine infusion reached its maximum rate within 3 hrs after the start of the infusion and remained approximately constant for 2 hrs or more Atropine was administered as a single i v injection (0 5 mg of atropine sulphate per 1 ml saline) 3 hrs after the start of the reserpine infusion the injection being completed in less than 1 min The injection of a corre sponding volume of saline did not affect acid secretion It was observed during the course of the study that the infusion of reserpine tended to reduce the secretory response to a subsequent infusion 3 to 5 days later Experiments on the same animal were therefore performed at intervals of at least one week except in reserpinized cats (see below) in which shorter intervals were allowed

In the nonvagotomized cats the amounts of reserpine infused i v ranged from 0 010 to 0 030 mg per kg b w for 15 min and were individually adjusted to produce a submaximal secretory response of acid Unless otherwise stated the total dos for each cat was kept constant throughout the series despite fluctuations in b w In the anesthetized cats 0 060 or 0 080 mg per kg was infused for 15 min The secretory rate was in all experiments far from the maximal secretory capacity of the cat s stomach (*cf* Emås 1960 1963) The doses of atropine (as sulphate) ranged from 0 05 to 0 5 mg per kg of b w

Basal secretion was defined as meq of total acid secreted during the hour before the infusion of reserpine The reserpine treatment of ordinary (nonanesthetized) gastric fistula cats for 3 days or more elevates the basal gastric secretion of acid (Emås 1963) correction was made for this in accordance with the principles previously appl ed (Emås 1963) The 5 hour secretory response to a single infusion of reserpine was accordingly defined as meq of total acid secreted during 5 hrs from the start of the infusion minus 5 times the 1 hr basal secretion

The reduction in acid output following atropine was calculated as the difference between the mean 15 min output of total acid (uncorrected) during the 30 min period before the injection of atropine (control level) and that during the 30 min period starting 1 hr after injection In the statistical analysis (see below) the differences were compared with cor responding differences in the experiments with reserpine alone on the same animal

In 2 ordinary gastric fistula cats secretion was recorded for 12 hrs following the infusion of reserpine In about half the number of experiments on each animal the fluid loss caused by the hypersecretion was partially compensated for by the continuous i v infusion of saline at a rate of 0 11 ml/min (infusion pump) The saline infusions started in most experiments 6 hrs after reserpine and continued for at least 4 hrs

The vehicle for Serpedin® in a volume corresponding to that for reserpine was infused i v for 15 min in 4 or 5 expts on each of 2 nonanethetized cats no influence on basal acid secreti n was observed during the subsequent 5 or 6 hrs in any experiment

Reserpine Treatment

Reserpin was injected i m in a dose of 0 10 mg per kg b w every 24 hrs for periods of usually 3 to 11 days (mean 8 days) After reserpine treatment for 3 days or more the animals are referred to as reserpinized (Emås 1963) The secretory studies were carried out every 2nd or 3rd day on the reserpinized animal each experiment starting 20 to 24 hrs after the previous reserpine injection On days when experiments with reserpine were performed the regular i m dose of reserpine was reduced to obtain a total daily dose of 0 10 mg per kg Each animal was subjected to 1 to 4 periods of reserpinization Further details have been given elsewhere (Emås 1963)

Experiments performed 24 hrs after a single i m injection of 0 10 mg of reserpine per kg (reserpine pretreatment) are accounted for separately

Evaluation of Data

The effects of atropine reserpine treatment sympathectomy and vagotomy on the secretory responses to single reserpine infusions were evaluated by current methods for the analysis of variance (Snedecor 1956) or — in unsymmetrical series of experiments on 2 or more cats — by a modified procedure for the analysis of variance (Snedecor 1956 Chapt 12 Sections 14 and 15)

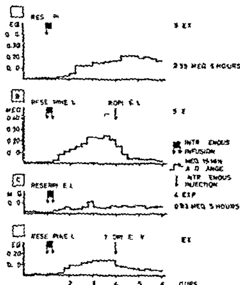


Fig 1 Basal gastric secretion and secretory response (uncorrected) to a single i v infusion of reserpine in a nonanesthetized gastric fistula cat (no 86)

A Before vagotomy Dose of reserpine 0 025 mg per kg of b w for 15 min Corrected mean secretory response 2 55 meq per 5 hours

B Before vagotomy Effect of i v atropine (0 50 mg per kg of b w) on the secretory response Dose of reserpine as in A

C After vagotomy Dose of reserpine as in A Corrected mean secretory response 0 93 meq per 5 hours

D After vagotomy Effect of i v atropine (0 05 mg per kg of b w) on the secretory response Dose of reserpine 0 050 mg per kg of b w for 15 min

Results

1 Experiments on Ordinary Gastric Fistula Cats

Secretory Response to a Single Reserpine Infusion

11 cats were used and 4 or 5 expts were performed on each animal After 30 to 60 min from the start of the reserpine infusion (0 010 to 0 030 mg per kg b w) acid secretion gradually increased reaching about maximum rate during the 3rd post infusion hour and remaining then almost constant for 2 to 3 hrs before slowly subsiding The mean responses of 2 representative cats are shown in Fig 1 A and 2 A The mean peak output of total acid varied among the 11 cats from about 0 10 to 0 40 meq per 15 min these variations being due to the different reserpine doses employed and to the different susceptibility of the cats to reserpine During the period of maximum secretion the curves for acid output in the experiments on the same animal had an almost horizontal course although at different levels Due to the small variation in acid output during maximum secretion within experiments the ranges in the figures reflect mainly the differences in secretory levels as between experiments

In 2 cats investigated mean acid output during the 12th hour after the infusion of 0 020 (Fig 2 A) or 0 010 mg (4 expts) of reserpine per kg amounted to about 50 per cent of maximum or 0 10 meq of total acid per 15 min The continuous i v infusion of saline for 4 hrs or more did not influence the slow decline in acid secretion

Effect of Atropine on the Secretory Response to a Single Reserpine Infusion

Following i v atropine inhibition appeared within 15 min reached its maximum in about 60 min and remained throughout the experiments 2 or 3 hrs The inhibition of acid secretion was never complete In 3 cats (no 81 82 and 86 0 50 mg of atropine per kg reduced the mean 15-min output of total acid by 0 18 2 expts 0 17 (4 expts)

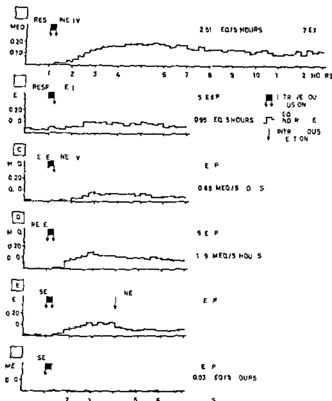


Fig 2 Basal gastric secretion and secretory response (uncorrected) to a single i v infusion of reserpine in a nonanesthetized gastric fistula cat (no 98)

A Before vagotomy Dose of reserpine 0 020 mg per kg of b w for 15 min Corrected mean secretory response (during the period from 1 to 6 hours 2 51 meq per 5 hours

B During reserpine treatment Preparation of the animal and dose of reserpine as in A Corrected mean secretory response 0 95 meq per 5 hours

C After vagotomy Dose of reserpine as in A Corrected mean secretory response 0 88 meq per 5 hours

D After vagotomy Dose of reserpine 0 040 mg per kg of b w for 15 min Corrected mean secretory response 1 79 meq per 5 hours

E After vagotomy Effect of i v atropine (0 25 mg per kg of b w) on the secretory response Dose of reserpine as in D

F During reserpine treatment Preparation of the animal and dose of reserpine as in D Corrected mean secretory response 0 03 meq per 5 hours

and 0 20 meq (Fig 1 B) or 75 to 83 per cent (mean control levels 0 24 0 22 and 0 24 meq) In 2 other cats (no 84 and 85) 0 25 mg of atropine per kg reduced the mean acid output by 0 20 meq or 65 per cent (4 expts mean control level 0 31 meq) and by 0 14 meq or 70 per cent (5 expts mean control level 0 20 meq) The inhibition following 0 50 and 0 25 mg per kg was highly significant ($P < 0\,001$) After 0 10 mg of atropine per kg (cat 89) the mean reduction was 0 12 meq of total acid ($P \sim 0\,05$ almost significant) or 55 per cent (2 expts mean control level 0 22 meq) In 3 to 5 expts with reserpine alone on each animal there was no apparent decline in mean acid output during the 4th and 5th hours after reserpine

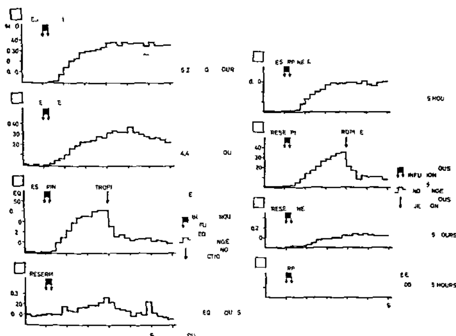


Fig 3 (left) Basal gastric secretion and secretory response (uncorrected) to a single i v infusion of reserpine (0 030 mg per kg of b w for 15 min) in a nonanesthetized gastric fistula cat (no 89)

A After preganglionic sympathectomy Corrected mean secretory response (during the period from 1 to 6 hours) 5 25 meq per 5 hours

B After pre and postganglionic sympathectomy Corrected mean secretory response 4 47 meq per 5 hours

C After pre and postganglionic sympathectomy Effect of i v atropine (0 10 mg per kg of b w) on the secretory response

D During reserpine treatment Preparation of the animal as in B Corrected mean secretory response 1 32 meq per 5 hours

Fig 4 (right) Basal gastric secretion and secretory response (uncorrected) to a single i v infusion of reserpine (0 030 mg per kg of b w for 15 min) in a nonanesthetized gastric fistula cat (no 71)

A After pre and postganglionic sympathectomy Corrected mean secretory response 4 11 meq per 5 hours

B After pre and postganglionic sympathectomy Effect of i v atropine (0 10 mg per kg of b w) on the secretory response

C After pre and postganglionic sympathectomy and vagotomy Corrected mean secretory response 1 70 meq per 5 hours

D During reserpine treatment Preparation of the animal as in C Corrected mean secretory response 0 00 meq per 5 hours

Secretory Response to a Single Reserpine Infusion during Reserpine Treatment

With the 3 cats used (no 98 155 and 162) reserpinization increased the mean 1 hr basal output of total acid by 0 03 to 0 11 meq

In cat 98 reserpinization reduced the mean 5 hr response to a single reserpine infusion by 1 56 meq of total acid (Fig 2 A and B) or 6? per cent The mean response

to 0 030 mg of reserpine per kg amounted in cat 155 to 1 87 meq per 5 hrs (4 expts) in the nonreserpinized state and 1 51 meq (4 expts) in the reserpinized state and in cat 162 to 5 47 (5 expts) and 2 40 meq (5 expts) respectively Reserpinization thus reduced the mean response of cats 155 and 162 by 19 and 56 per cent The responses of the nonreserpinized and reserpinized cats differed highly significantly ($P < 0\ 001$) The size of the reduction was not related to the length (3 days or more) of the reserpinization period

The figures for percentage reduction were approximately the same when calculated on acid output during the 5th post infusion hour instead of on the 5 hr secretory response

B Experiments on Sympathectomized Gastric Fistula Cats

The experiments on cat 39 started 4 months after preganglionic sympathectomy and were carried out within a period of 2 months and on cat 71 5 months after pre and postganglionic sympathectomy and were completed in 6 months On cat 89 experiments were performed both after preganglionic and after pre and postganglionic sympathectomy The former experiments started 15 days the latter 8 months after the operation and were completed in 5 and 8 months respectively

Secretory Response to a Single Reserpine Infusion

The curves for acid output following the infusion of reserpine (0 075 or 0 030 mg per kg) had the same shape in the preganglionically (no 39 and 89) and the pre and post ganglionically sympathectomized cats (no 71 and 89) as in ordinary gastric fistula cats and the 5 hour responses fell within the ranges of the latter The mean output of total acid reached in cat 39 a maximum level of 0 25 meq per 15 min (4 expts) during the 3rd hour after reserpine (0 025 mg per kg) remaining then almost constant for the last 2 hrs The mean responses of cat 89 and 71 are shown in Fig 3 A and B and Fig 4 A respectively In cat 89 the responses after preganglionic (Fig 3 A) and after pre and postganglionic sympathectomy (Fig 3 B) did not differ significantly ($P > 0\ 05$)

Effect of Atropine on the Secretory Response to a Single Reserpine Infusion

In a preganglionically sympathectomized cat (no 39) 0 50 mg of atropine per kg reduced the mean 15 min output of total acid by 0 11 meq ($P < 0\ 05$) or 58 per cent (4 expts mean control level 0 19 meq) while in 2 pre and postganglionically sympathectomized cats (no 71 and 89) 0 10 mg of atropine per kg reduced the mean acid output by 0 24 meq or 69 per cent (Fig 4 B) and by 0 28 meq or 67 per cent (Fig 3 C) a highly significant ($P < 0\ 001$) reduction Complete inhibition was never observed The experiments with reserpine alone are reported above

Secretory Response to a Single Reserpine Infusion during Reserpine Treatment

Experiments were performed on cat 89 after preganglionic and after pre and post ganglionic sympathectomy and gave similar results as those on ordinary gastric fistula cats

After preganglionic sympathectomy reserpinization (2 expts) increased the mean 1 hr basal output of total acid by 0 16 meq but reduced the mean 5 hr response to i v reserpine by 4 48 meq ($P < 0\ 001$) or 85 per cent The mean response of the non reserpinized animal is illustrated in Fig 3 A

TABLE I Mean 5 hour secretory response to intravenous infusions of reserpine in nonanesthetized gastric fistula cats before and after bilateral vagotomy

		Before vagotomy		After vagotomy		Decrease	
Cat no	Reserpine in mg/kg	No of expts	Response in meq	No of expts	Response* in meq	in meq	in per cent
29	0.010	4	1.92 0.77—2.96	5	0.73 0.49—0.92	1.19	62
41	0.010	4	1.39 0.58—2.20	4	0.48 0.17—1.25	0.91	65
86	0.025	5	2.55 1.98—3.18	4	0.93 0.36—1.36	1.62	64
*98	0.020	7	2.51 1.83—2.90	4	0.88 0.45—1.39	1.63	65
155	0.030	4	1.87 1.60—2.21	1	0.30	1.57	84
Pre- and postganglionically sympathectomized							
*71	0.030	5	4.11 2.03—5.69	5	1.70 1.19—2.43	2.41	59

Correction made for basal secretion
Range of secretory responses
Illustrated in Fig 1 A and C
* Illustrated in Fig 2 A and C
Illustrated in Fig 4 A and C

After pre and postganglionic sympathectomy the increase in the mean 1 hr basal output of total acid during reserpinization amounted to 0.35 meq and the reduction of the mean response to i.v. reserpine to 3.15 meq or 70 per cent (Fig 3 B and D) This reduction was significant ($P < 0.01$)

C Experiments on Vagotomized Gastric Fistula Cats

In 8 of 9 cats the experiments with reserpine started within less than 3 months and in the remaining cat 6 months after complete vagotomy The experiments were completed in 4 months except for cat 94 in which the last experiment was performed 16 months after vagotomy Cat 71 had been pre and postganglionically sympathectomized before the vagotomy

Secretory Response to a Single Reserpine Infusion

Reserpine (0.010 to 0.060 mg per kg) produced usually within 1 hr a gradual increase of gastric acid secretion (Figs 1 C 2 C, and 4 C) in all 9 vagotomized cats The maximum rate of acid secretion was reached during the 3rd or 4th post infusion hour and then remained approximately constant or declined slightly during the subsequent 2 to 3

hrs. The mean peak acid output varied among the animals from about 0.05 to 0.15 meq per 15 min.

In the 6 cats investigated, vagotomy reduced the mean 5-hr response (Table I) by 0.91 to 2.41 meq or 59 to 84 per cent. This reduction was highly significant ($P < 0.001$). The experimental data of cat 71 were not included in the statistical analysis. Fig. 1, 2 and 4 (A and C) show the mean responses of 3 cats before and after vagotomy.

Effect of Atropine on the Secretory Response to a Single Reserpine Infusion

In 3 vagotomized cats 0.25 mg of atropine per kg reduced the reserpine induced acid secretion highly significantly ($P < 0.001$): in cat 29 the mean 15 min output of total acid (0.015 mg of reserpine per kg) was reduced by 0.03 meq or 27 per cent (3 expts; mean control level 0.11 meq); in cat 98 by 0.06 meq or 52 per cent (Fig. 2 E) and in cat 165 (0.060 mg of reserpine per kg) by 0.07 meq or 62 per cent (5 expts; mean control level 0.11 meq). In one cat investigated, 0.05 mg of atropine per kg reduced acid output by 0.09 meq ($P < 0.01$) or 61 per cent (Fig. 1 D). 4 or 5 expts. were performed with reserpine alone on each animal.

Secretory Response to a Single Reserpine Infusion during Reserpine Treatment

The 5-hr secretory response to i.v. reserpine was determined in 4 cats, nonreserpinized and reserpinized. Reserpinization had no influence on the basal output of total acid but eliminated the secretory response to i.v. reserpine in all cats. The mean 5 hr responses of cat 71, nonreserpinized and reserpinized, are shown in Fig. 4 C and D and of cat 98 in Fig. 2 D and F. The mean response in cat 94 (0.030 mg of reserpine per kg) amounted in the nonreserpinized state to 3.93 meq (6 expts.) and in the reserpinized state to 0.06 meq (5 expts.), and in cat 165 (0.060 mg per kg) to 1.77 meq (5 expts.) and less than 0.01 meq (4 expts.), respectively.

The variation in responses was much greater in the nonreserpinized than in the reserpinized animal, making the ordinary analysis of variance inappropriate. There was no doubt, however, as to the effect of reserpinization, since the reduction was very uniform and pronounced.

Reserpine treatment for 3 days or more reduced or even eliminated (in the vagotomized cats) the secretory response to i.v. reserpine. In a series of experiments the 5 hr secretory responses to i.v. reserpine were determined also 24 hrs after a single i.m. injection of 0.10 mg of reserpine per kg (pretreatment). In one ordinary gastric fistula cat (no. 98) reserpine pretreatment reduced the mean response (2 expts.) by 0.72 meq or 29 per cent, while in the other (no. 162) no reduction occurred (3 expts.). Reserpinization for 3 days reduced the mean response in these animals by about 60 per cent (see Section A). In cat 89 (pre- and postganglionically sympathectomized) a single reserpine injection reduced the mean response (6 expts.) to i.v. reserpine by 1.65 meq (P 0.05, not significant) or 37 per cent, while reserpinization for 3 days reduced the mean response by 70 per cent (see Section B). In 11 expts. on 3 vagotomized cats reserpine pretreatment reduced the response to i.v. reserpine by 1.31 (cat 98) to 2.09 meq (cat 94) or 53 (cat 94) to 76 per cent (cat 165) — a highly significant (P 0.001) reduction — but in contrast to reserpinization (see Section C) it never actually abolished the response.

In the nonvagotomized cats, pretreatment with reserpine increased basal acid secretion to about the same extent as reserpine treatment for 3 days or more. Reserpine pretreatment did not alter basal secretion in the vagotomized cats.

D Experiments on Anesthetized Gastric Fistula Cats Subjected to Vagotomy and Resection of Gastrin Releasing Regions

Secretory Response to a Single Reserpine Infusion

The 1 hr basal secretion contained no titratable free acid and total acid amounted to 0 01 meq or less

A single i v reserpine infusion (0 060 or 0 080 mg per kg) elicited acid secretion within 1 hr in all 7 cats The curves for total acid output had in principle the same shape as those obtained from the nonanesthetized cats with vagally denervated stomachs The peak output of total acid was reached in the 3rd or 4th post infusion hour and varied among the cats from approximately 0 05 to 0 30 meq per 15 min The 5-hr response ranged from 0 43 to 4 81 meq of total acid (mean 2 20 meq)

Discussion

Intravenous reserpine has been shown to stimulate gastric acid secretion in humans (Beman Knoll and DeLor 1955 Clark and Schneider 1955 Haverback *et al* 1955 Krogsgaard 1955) nonanesthetized dogs (Barrett Rutledge and Rogie 1954) and anesthetized cats (Gaitonde Satoskar and Mandrekar 1960) In the present study on nonanesthetized cats a single i v reserpine infusion elicited a gastric secretion of acid which appeared after a delay of 30 to 60 min and lasted for at least 12 hrs

In agreement with previous reports (Emås 1961 Schapiro and Woodward 1961) the sympathetic nerves to the stomach appear to be inessential to the stimulating effect of reserpine on acid secretion since the secretory response to reserpine was about as great in sympathectomized as in ordinary gastric fistula cats The difficulty of performing a complete and permanent sympathetic denervation of the stomach has been emphasized previously (Emås 1964 a)

Injected reserpine stimulates acid secretion in the vagally denervated stomach of humans (Kirsner and Ford 1957 Rider Moeller and Gibbs 1957 Schneider and Clark 1957) dogs (Schapiro and Woodward 1961 Castiau and Reuse 1962) and rats (Kim and Shore 1963) Only a few data have been reported illustrating the effect of vagotomy on the secretory response to reserpine Schneider and Clark (1957) reported that vagotomy did not significantly alter the acidity of the response in 2 human subjects Kim and Shore (1963) obtained a profound reduction of the acid response in non anesthetized rats which they ascribed to the vagotomy greatly inhibiting the reserpine induced release of gastric histamine It has been suggested that a parasympathetic mechanism is implicated in the stimulating action of reserpine on acid secretion since anticholinergic drugs reduced the acid response in nonanesthetized dogs with vagally innervated stomachs (Barrett Rutledge and Rogie 1954 La Barre and Lieber 1957) Corresponding studies on humans however have given varying results (for references see Levrat and Lambert 1962) and in anesthetized dogs even large doses of atropine (2 mg per kg i v) failed to reduce the reserpine induced secretion (Castiau and Reuse 1962) In the present investigation vagotomy reduced the response to reserpine in nonanesthetized cats by 59 to 84 per cent and about as large a reduction was produced by atropine (0 10 to 0 50 mg per kg) in nonvagotomized cats The reduction seems mainly to reflect a reduced excitability of the HCl secreting cells due to the elimination or inhibition of vagal sensitizing impulses to the parietal cells (Emås 1964 b) since in 3 out of 4 cats used also in a previous study (Emås 1964 b) vagotomy

produced approximately the same percentage reduction of the responses to infused histamine and infused gastrin extracts A blockade or reduction of a vagal and gastrin releasing effect of reserpine (see next paragraph) might however also have contributed to the reduction Neither vagotomy nor atropine abolished the secretory response to reserpine completely The stimulating effect of a single reserpine infusion on acid secretion therefore differs from the effect of reserpine treatment on acid secretion (Emås 1964 b) and from that of reserpine on antral and duodenal gastrin activity (Emås and Fyro 1965) in that it does not require intact vagal nerves In nonanesthetized cats however intact vagal nerves are essential for a single reserpine infusion to exert an optimal stimulating effect

A previous study (Emås and Fyro 1965) demonstrated that 0 10 mg of reserpine per kg i m produced a reduction of antral gastrin activity in nonvagotomized cats while no measurable reduction occurred in nonvagotomized cats after 0 030 mg per kg or in vagotomized cats after 0 10 mg per kg The secretory response of the non vagotomized cats to the relatively small amounts of reserpine infused in the present study (0 010 to 0 030 mg per kg) could therefore be due at least in part to an action of reserpine that does not involve gastrin release In the vagotomized cats the vagal preganglionic neurons can be excluded and gastrin seems an unlikely mediator for the stimulating action of a single reserpine infusion on acid secretion An action of reserpine on acid secretion independently of the vagal nerves and the gastrin mechanism was supported also by the finding that a single reserpine infusion elicited a significant response of acid in anesthetized vagotomized cats in which the gastrointestinal regions known or suspected to contain gastrin had been resected Moreover there was no close parallelism in nonanesthetized cats between the effects of reserpine treatment or pre treatment on the secretory response to i v reserpine and on the antral gastrin activity In nonvagotomized cats reserpine treatment significantly reduced both the secretory response to i v reserpine — this has been observed also in two human subjects (Pachrach 1959 Haverback and Wirtschafter 1962) — and the antral gastrin activity (Emås and Fyro 1965) but increased the susceptibility of the HCl secreting cells to exogenous histamine and gastrin (Emås 1963 1964 a) In vagotomized cats reserpine treatment abolished the response to i v reserpine without altering either the antral gastrin activity (Emås and Fyro 1965) or the susceptibility of the HCl secreting cells to histamine and gastrin (Emås 1964 b) Pretreatment with reserpine 24 hours before the experiment on the other hand did not significantly alter the acid response to i v reserpine in nonvagotomized cats although it reduced antral gastrin activity as much as 2 or 4 days of treatment (Emås and Fyro 1965) It decreased the response significantly however in vagotomized cats

The latent period and long duration of the secretory response to reserpine in addition to the reduction of the response by reserpine treatment suggest that the non vagal and non-gastrin component of reserpine action elicits acid secretion by an intermediate mechanism. The acid response is reported to persist after adrenalectomy in dogs (*Schapiro and Woodward 1961* Castiau and Reuse 1962) but no experimental data were presented The persistence of the response demonstrates that the adrenals are not essential for the stimulatory action of reserpine on acid secretion It offers however no information concerning a cooperation of the adrenals since the vagal innervation to the stomach was left intact making gastrin release possible (Emås and Fyrö 1965) Reserpine has been shown to reduce the histamine content of various tissues (Waalkes Coburn and Terry 1959 Haverback and Wirtschafter 1962 Moran and Westerholm

1963) The finding of Kim and Shore (1963) could indicate that reserpine caused a release of gastric histamine in rats mainly by a vagal action Whether histamine is the mediator for the non vagal and non gastrin component of reserpine action cannot be excluded however

Barrett *et al* (1955) suggested that reserpine evokes gastric acid secretion by stimulating the parasympathetic ganglia since an anticholinergic and a ganglionic blocking drug inhibited the reserpine induced secretion in the vagally denervated (Heidenhain) gastric pouch of dogs In this pouch preparation the vagal supply of the antral and duodenal mucosa is left intact which makes gastrin release by reserpine possible (Emås and Fyro 1965) Kim and Shore (1963) reported that the ganglionic blocking drug chlorisondamine was more effective than vagotomy in reducing the acid response of nonanesthetized rats to reserpine The small but significant reduction of reserpine induced acid secretion by atropine in the vagotomized cats might appear to support the suggestion of Kim and Shore that part of the secretory response to reserpine is produced by ganglionic stimulation However this hypothesis for reserpine action seems difficult to reconcile with the finding that the reserpine treatment of vagotomized cats abolished the secretory response to a single reserpine infusion but did not alter the excitability of the HCl secreting cells (Emas 1964 b) Furthermore in vagotomized cats the acid secretion elicited by a single reserpine infusion and by a continuous i v infusion of histamine or of gastrin preparations was susceptible to inhibition by atropine and reserpine and histamine induced secretion were about equally reduced by 1 mg of chlorisondamine (Ecolid®) per kg b w i v (Emås unpublished observations) The two inhibitors were tested at approximately the same submaximal secretory rate Since cholinergic excitation increases the responsiveness of the HCl secreting cells to other stimuli (for references see Emås 1964 b) the reductions by atropine and chlorisondamine in the vagotomized cats can possibly be ascribed to the blockade of a sensitizing effect of the postganglionic cholinergic neurons on the HCl secreting cells rather than to a blockade of the effect of ganglionic stimulation

To conclude the present author agrees with the view that a single reserpine infusion elicits acid secretion by intermediate mechanisms and not by an action directly on the HCl secreting cells Evidence of a vagal action of a relatively large dose of reserpine (0 10 mg per kg) causing gastrin release has previously been published (Emås and Fyro 1965) The present investigation demonstrates that an intact sympathetic and vagal supply of the stomach is not necessary for reserpine (0 01 to 0 03 mg per kg) to stimulate acid secretion but that intact vagal nerves are necessary for reserpine to exert its optimal stimulatory effect The reduction of the response by atropine in the nonvagotomized cats and by vagotomy is assumed to be due mainly to the inhibition of vagal sensitizing impulses to the HCl secreting cells (Emås 1964 b) and the small reduction by atropine in the vagotomized cats to the inhibition of a sensitizing effect of acetylcholine released from the postganglionic cholinergic neurons In favour of a secretagogue action of reserpine which is not mediated by the vagal nerves and the gastrin mechanism are the findings (1) that the i v infusion of relatively small amounts of reserpine elicited acid secretion in both nonvagotomized and vagotomized cats but produced no measurable reduction of antral gastrin activity in nonvagotomized cats and 3 to 10 times these amounts produced no measurable reduction of activity in vagotomized cats (Emås and Fyrö 1965) (2) that 3 days or more of reserpine treatment reduced and abolished the secretory response to i v reserpine in nonvagotomized and vagotomized cats respectively but reduced antral gastrin activity only in the nonvagotomized cats (Emås and Fyro

1965) (3) that reserpine pretreatment of nonvagotomized cats did not significantly alter the secretory response to i v reserpine but reduced antral gastrin activity as much as 2 or 4 days of reserpine treatment (Emås and Fyro 1965) and (4) that i v reserpine elicited acid secretion in anesthetized cats subjected to vagotomy and resection of regions known or suspected to contain gastrin

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Acta physiol scand 1965 64 238—244

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Renal Clearances of Inulin, Polyfructosan-S and a Polyethylene Glycol (PEG 1,000) in the Rat

By

FREDRIK BERGLUND[1]

Received 22 October 1964

Abstract

Berglund F *Renal clearances of inulin polyfructosan S and a polyethylene glycol (PEG 1 000) in the rat* Acta physiol scand 1965 64 238—244 The renal clearances of inulin polyfructosan S and PEG 1 000 were measured in the rat by means of a constant infusion technique The inulin/PEG 1 000 clearance ratio in 10 rats averaged 0.81 and polyfructosan S/PEG 1 000 ratio in 10 rats averaged 0 87 The clearance ratios differed signicantly from unity and were not influenced by phlorizin The restricted filterability of the two polysaccharides in the rat is attributed to the pore size of the glomerular membrane and a relationship between pore size and animal size is suggested

The clearance of inulin has generally been considered to be the most reliable measure of glomerular filtration rate (GFR) since its introduction for this purpose in 1934 In certain species the clearance of other unrelated substances may equal that of inulin and thus presumably equal GFR In the dog this applies to creatinine (Shannon 1936) ferrocyanide ion Berliner Kennedy and Hilton 1950) glucoseamine (Carter and Peters 1958) polyethylene glycols of molecular weight up to 4 000 (Shaffer Critchfield and Carpenter 1948 and dextran of molecular weight up to 4 000 (Wallenius 1954)

In a recent attempt to measure renal tubular reabsorption of inorganic sulfate in the rat, a reabsorption secretion pattern was obtained evidently because inulin clearances were lower than GFR When GFR was measured with low molecular weight polyethylene glycol (PEG 1 000 or PEG 400) instead of inulin a regular Tm pattern was obtained for sulfate reabsorption (Berglund 1964)

In the present paper the clearances of two fructans inulin and polyfructosan S are compared with the clearance of PEG 1 000 in the rat

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Methods

Clearance experiments were done on unanesthetized male blackhooded rats by a constant infusion technique (Berglund 1964) Each experiment consisted of six clearance periods Test substances were inulin derived from chicory (L Light & Co Ltd) polyfructosan S (Laevosan Gesellschaft) and PEG 1 000 (Union Carbide)

Inulin and polyfructosan-S were analysed by the method of Heyrovsky (1956) and PEG 1,000 by the method of Hydén (1955) as modified by Berglund (1964)

Results

Clearances and clearance ratios are listed in Table I The inulin clearance (0 78 ± 0 14 ml/min/100 g) was significantly higher than reported by earlier authors (cf Smith 1951 Fingl 1952) with the exception of Peters (1959) (0 70 ± 0 21 ml/min/100 g)

Inulin/PEG 1 000 clearance ratios averaged 0 81 and polyfructosan S/PEG 1 000 clearance ratios averaged 0 87 The difference between these ratios is not statistically significant but both ratios differed significantly from unity The inulin/PEG clearance ratio was well below 1 0 even when arterial blood samples were used for analysis (exp 334)

In one rat inulin/PEG 1 000 clearance ratios were measured in two consecutive experiments using non boiled inulin (exp 333) and inulin which had been kept in boiling water for 1 hour (exp 332) The boiled inulin showed a higher clearance ratio than the non boiled

Phlorizin (exp 307) did not raise a low polyfructosan S/PEG 1 000 clearance ratio significantly This should rule out tubular reabsorption of polysaccharide as a possible source of error

Discussion

The low polysaccharide/PEG 1 000 clearance ratios in the rat may be related to various factors, such as molecular weight and molecular inhomogeneity of the test substances as well as the average pore size of the glomerular membranes These points will be discussed especially in relation to inulin

Inulin is a D fructose polymer or fructan containing small amounts of D glucose probably in a sucrose type linkage at the reducing end of the molecule It forms the major polysaccharide in tubers of many *Compositae* Most commercial inulin seems to derive from dahlia (*Dahlia sea*) but at least three brands (L Light & Co Ltd Laevosan-Gesellschaft E Merck A G) derive from chicory (*Cichorium Intybus*)

For the molecular weight of inulin the figure 5100 (Westfall and Landis 1936 is quoted in textbooks of renal physiology (Smith 1951 Pitts 1963) in spite of higher figures in the literature since 1936 (Table II Molecular weight does not seem to vary significantly between inulins from different genera Highly divergent molecular weights may however be obtained before and after fractionation e g with ethanol The figures given by Lenaka (1951) and by Vink (1960) are especially noteworthy in this respect (cf Table II and indicate a considerable degree of molecular inhomogeneity of inulin This has also been demonstrated by paper electrophoresis and by differential precipitation with ethanol Bassir 1956 The continuous fall in inulin clearance after a single intravenous injection in man may also be explained by inhomogeneity high molecular weight fractions supposedly being excreted slower than the low molecular weight fractions Ferguson *et al* 1950 Barnard Bassir and Hough 1955)

TABLE I Clearance ratios (inulin/PEG 1 000 or polyfructosan S/PEG 1 000) in rats

Exp	Weight g	Plasma fructan mg %	Plasma PEG mg	Fructan clearance ml/min/100 g	PEG clearance ml/min/100 g	Fructan/PEG clearance ratio
Fructan = Inulin						
2[illegible]9	341	54	82	0.61	1.03	0.61
260	25[illegible]	63	103	0.71	0.98	0.72
327	240	33	86	1.04	1.31	0.81
332	2[illegible]7	72	Inulin in 100 C water bath for 60 min Same rat as exp 333			
			134	0.44	0.51	0.87±0.0[illegible]
333	2[illegible]7	41	97	0.67	0.94	0.72±0.07
334	2[illegible]0	44	75	0.39	0.60	0.65
339	300	36	62	0.88	1.0[illegible]	0.83
340	335	41	71	0.74	0.73	1.03
341	345	3[illegible]	[illegible]9	0.85	0.81	1.0[illegible]
342	360	24	88	0.65	0.82	0.81
343	3[illegible]	31	55	0.87	1.0[illegible]	0.83
Fructan = Polyfructosan S						
30[illegible]	[illegible]	56	145	0.[illegible]9	0.81	0.8[illegible]
307	228	88	67	0.84	1.30	0.64
		Phlorizin 44 mg/kg i v		0.7[illegible]	1.11	0.67
308	300	72—114	106	0.93	1.14	0.83
310	248	64	149	0.70	1.06	0.6[illegible]
311	290	44	101	1.13	1.22	0.89
312	2[illegible]0	44	8[illegible]	1.17	1.22	0.9[illegible]
313	290	4[illegible]	100	1.12	1.13	1.00
315	2[illegible]5	[illegible]4	200	1.01	0.99	1.04
316	275	9[illegible]	160	0.73	0.82	0.89
338	34[illegible]	47	56	1.07	1.15	0.93

Notes Exp 307 phlorizin data not included in statistical treatment
Exp 3[illegible] dahl a inulin (British Drug Houses Ltd)
Exp 332 included only in statistical treatment [illegible]
Exp 334 anesthesia with Inactin® Na [illegible]-ethyl [illegible]-(1 methyl propyl) th barbiturate
Blood samples from carotid artery Included only in statistical treatment 4

Statistical treatment (means ± standard deviations)

1 Clearance of inulin 0.78 ± 0.14 ml/min/100 g n = 9
2 Clearance of polyfructosan S 0.91 ± 0.19 ml/min/100 g n = 10
3 Clearance of PEG 1 000
inulin series 0.9[illegible] ± 0.19 ml/min/100 g n = [illegible]
polyfructosan S series 1.03 ± 0.17 ml/min/100 g n = 10
combined 1.03 ± 0.18 ml/min/100 g n = 19

TABLE II Molecular weight of inulin Data from 1936 and later

Source of inul n	Method	Mol wt.	Reference
Dahlia recrystallized twice	Thermoelectric vapor pressure	4 457	Westfall and Landis 1936
Recrystallized 5 times		5 101	
Inula helenium repeated purification	Thermoelectric vapor pressure	7 777	Bezzi 1939
Blue Danube dahlia tubers	Analys s of hydrolysis products	6 150	Hirst et al 1950
Taraxacum officinal	Glucose analysis with notatin	6 300	Palmer 1951
Dahlia rapidly crystallizing		6 800	
Dahlia slowly crystallizing		7 290	
Inula h l nium		6 800	
Kahlbaum original	Osmometer cellophane membrane	5 200	Uenaka 195
Repeated ethanol fractionation		7 000	
Cich um Intybus before ethanol fractionation	Osmometer v scose membrane	3 800	V nk 1960
3 d prec pitate		4 900	
12th p ecipitate		7 700	

4 Clearance rat os
a) inulin/PEG 1 000 0 81 ± 0 14 n = 10
b) polyfructosan S/PEG 1 000 0 87 ± 0 13 n = 10
Comparison a) vs b) p > 0 20
Comparison a) vs un ty p < 0 005
Comparison b) vs un ty p < 0 025
5 Exp 337 clearance rat o 0 87 ± 0 05 n = 6
Exp 333 clearance ratio 0 72 ± 0 07 n 6
Comparison exp 337 vs. 333 p < 0 001

TABLE I Clearance ratios (inulin/PEG 1 000 or polyfructosan S/PEG 1 000) in rats

Exp	Weight g	Plasma fructan mg %	Plasma PEG mg	Fructan clearance	PEG clearance	Fructan/PEG clearance ratio
				ml/min/100 g		
Fructan = Inulin						
259	341	54	82	0 61	1 03	0 61
260	255	63	103	0 71	0 93	0 72
327	240	33	86	1 04	1 31	0 81
332	257	72	Inulin in 100 C water bath for 60 min Same rat as exp 333			
			134	0 44	0 51	0 87±0 05
333	257	41	97	0 67	0 94	0 72±0 07
334	250	44	75	0 39	0 60	0 65
339	300	36	62	0 88	1 09	0 83
340	335	41	71	0 74	0 73	1 03
341	345	36	69	0 85	0 81	1 05
342	360	24	88	0 65	0 82	0 81
343	375	31	55	0 87	1 06	0 83
Fructan = Polyfructosan S						
305	245	56	145	0 69	0 81	0 85
307	228	88	67	0 84	1 30	0 64
		Phlorizin 44 mg/kg i v		0 75	1 11	0 67
308	300	72—114	106	0 93	1 14	0 83
310	248	64	149	0 70	1 06	0 66
311	290	44	101	1 13	1 2[illegible]	0 89
312	250	44	85	1 17	1 22	0 96
313	290	45	100	1 12	1 13	1 00
315	275	54	200	1 01	0 99	1 04
316	275	97	160	0 73	0 87	0 89
338	342	47	56	1 07	1 15	0 93

Notes Exp 307 phlorizin data not included in statistical treatment
Exp 327 dahlia inulin (British Drug Houses Ltd)
Exp 332 included only in statistical treatment 5
Exp 334 anesthesia with Inactin® Na 5-ethyl 5-(1 methyl propyl) thiobarbiturate Blood samples from carotid artery Included only in statistical treatment 4

Statistical treatment (means ± standard deviations)

1 Clearance of inulin	0 78 ± 0 14 ml/min 100 g	n = 9
2. Clearance of polyfructosan S	0 94 ± 0 19 ml min 100 g	n 10
3 Clearance of PEG 1 000		
inulin series	0 97 ± 0 19 ml min 100 g	n = 9
polyfructosan S series	1 08 ± 0 17 ml/min 100 g	n 10
combined	1 03 ± 0 18 ml/min/100 g	n = 19

TABLE II Molecular we ght of inulin Data from 1936 and later

Source of inulin	Method	Mol wt	Reference
Dahlia recrystallized twice	Thermoelectric vapor pressure	4 457	Westfall and I an I s 193f
Recrystallized 5 times		5 101	
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Blue Danube dahlia tubers	Analysis of hydrolysis produ ts	6 150	Hirst et al 13 0
Taraxacum officinale	Clucose-analysis with notatin	6 300	Palmer 1) 1
Dahlia rapidly crystallizing		6 800	
Dahlia slowly crystallizing		7 290	
Inula helenium		6 800	
Kahlbaum original	Osmometer cellophane membrane	5 200	[illegible]
Repeated ethanol fraction at on		7 000	
Cicho um Intybus before ethanol fractionation	Osmometer viscos membrane	3 800	[illegible]
3 d prec pitate		4 100	
12th precipitate		7 700	

4 Clearar ce ratios		
a) inul n PEG 1 000	0 81 ± 0 14	n – 10
b) polyfru tosa i-S PEG 1 000	0 87 ± 0 1	r 10
Comparison a) vs b)	p – 0 1	
Con pa ison a) vs unity	p < 0 001	
Comparison b) vs un ty	p < 0 02	
5 Exp 332 clearance ratio	0 87 ± 0 0	r – (
Exp 333 clearance rat o	0 2 ± 0 07	n (
Comparison exp 332 vs. 333	p < 0 01	

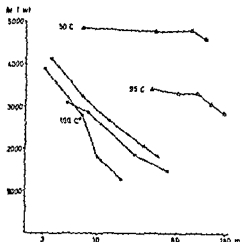


Fig 1 Depolymerization of inulin at different temperatures 100 C ebullioscopic measurements (Drew and Haworth 1928) 95 C and 50 C thermoelectric vapor pressure (Westfall and Landis 1936)

Commercial inulin contains both alkali stable inulin and inulin of smaller molecular weight susceptible to destruction with alkali (Cotlove 1954) The clearances of the two fractions seem to be equal in man (Walser Davidson and Orloff 1955, Davidson and Sackner 1963)

Careless preparation of inulin infusions may depolymerize the inulin molecule and increase inhomogeneity The ebullioscopic molecular weight data of Drew and Haworth (1928) indicate rapid depolymerization by boiling (Fig 1) This might affect the inulin clearance in two ways By reduction of molecular size the filterability and clearance would increase as seems to occur in the rat (Table I) On the other hand fructose molecules might be liberated (Vink 1950) and in the tubules these would be reabsorbed thus lowering the measured inulin clearance

Certain radioactive inulin derivatives (inulin C OOH and inulin OCH³) pass faster than ordinary inulin through cellophane tubing in dialysis or centrifugal pressure ultrafiltration evidently because of lower molecular weight (Chen Terepka and Lane 1963) Inulin C OOH also passes faster than ordinary inulin from the blood into the thoracic duct lymph of the dog (Chen and Lane 1964)

The multiple sources of inulin the variation of molecular weight between different preparations or solutions and the marked molecular inhomogeneity are severe drawbacks in the use of inulin for measuring GFR Furthermore inulin clearance is evidently not a valid measure of GFR in the rat

Polyfructosan S has recently been introduced as a substitute for inulin It is a fructan extracted from species of *Liliaceae* with a molecular weight around 2 756 (90 within 2 270—4 740) (Mertz and Sarre 1963) It is readily soluble in cold water and is alkali stable In man its clearance is identical to that of inulin and in the dog its clearance is equal to that of creatinine (Harth 1963) In the rat however its clearance is slightly higher than that of inulin but still significantly below that of PEG 1 000

Polyethylene glycols offer a number of advantages as test substances They are readily soluble in cold water The chain length of the molecules can be fairly well controlled through the synthetic process thereby giving a high degree of homogeneity (Flory 1940) Mixtures of different molecular weights can be fractionated by chromatography or with trichloroacetic acid in presence of barium ions It might therefore be possible

to "titrate the pore size in the glomeruli by using polyethylene glycols of various molecular weights

In the dog the clearances of polyethylene glycols of molecular weights from 400 up to 4 000 equal that of creatinine whereas a preparation of molecular weight 6 000 has a 25 per cent lower clearance (Shaffer Critchfield and Carpenter 1948) The clearances of different molecular weight PEG preparations in the rat are now being investigated

Glomerular pore size The pore size in the glomerular membranes may be measured by the molecular weight at which restricted permeation i e a lower clearance begins to occur The clearance of dextran equals GFR with molecular weights of dextran up to 4 000 in the dog (Wallenius 1954) but up to 15 000 in man (Arturson and Wallenius 1964) Pore size is evidently smaller in the dog than in man This is also indicated by experiments with "Levan a polyfructan prepared from Italian rye grass *Lolium multiflorum* with a mean molecular weight around 5 400 but containing fractions of molecular weight towards 9 000 (Beattie and Corcoran 1952) In man its clearance approximates GFR but in the dog its initial clearance is about 25 below GFR

In the rat both inulin and polyfructosan-S clearances are significantly lower than the clearance of PEG 1 000 evidently because the average pore size of the glomeruli is even smaller than in the dog A relationship between glomerular pore size and size of the animal might exist within mammals

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Skilful technical assistance was given by Mrs Mona Agren and Mrs Gunilla Friberg Polyfructosan S was generously supplied by Laevosan Gesellschaft Linz/Donau Austria

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Acta physiol scand 1965 64 245—250

From the Department of Physiology Faculty of Medicine Karolinska Institutet, Stockholm Sweden

The Effect of Dextran and Some other Colloids on the Suspension Stability of Blood from Different Species

By

R ELIASSON and U SAMELIUS BROBERG

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Abstract

Eliasson R. and U Samelius Broberg *The effect of dextran and some other colloids on the suspension stability of blood from different species* Acta physiol scand. 1965 64 245—250 — The effect of various dextran fractions and plasma proteins on the suspension stability of blood from different mammals has been studied in vitro The suspension stability was measured as erythrocyte sedimentation rate (ESR) according to Westergren s method None of the compounds caused a change in ESR of blood from cow and sheep The ESR of blood from cat and horse was increased by all compounds Rat rabbit dog pig and man formed an intermediate group

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The effect of dextran and other colloids on the suspension stability of washed human blood cells was extensively studied by Thorsén and Hint (1950) They demonstrated that the effect of the colloid on the ESR was a function of the concentration molecular weight and asymmetry of the colloid Below a certain molecular weight characteristic for each colloid there was no decrease in the suspension stability even at very high concentration This "critical molecular weight was about 60 000 for dextran

Although many investigations have been performed concerning the suspension stability of blood there has been relatively little attention paid to species differences

In a previous paper (Eliasson and Samelius-Broberg 1963) it was shown that a dextran preparation which clinically has proved to be a suitable plasma expander was not to be recommended in experiments on cats due to the severe decrease in the suspension stability evoked in this species Preliminary experiments revealed on the other hand that injection of rather high molecular weight dextran (M_w about 100 000) into sheep did not cause any increase in the ESR It was therefore thought to be of interest to further study the effect of dextran on the ESR in blood from various species In some experiments the effect of other macromolecules (albumin fibrinogen globulin) was also studied

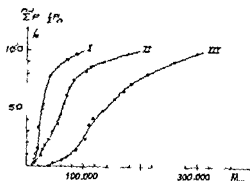


Fig 1 Molecular weight curves for 3 of the dextran fractions used
I Dextran 40 000
II Dextran 75 000
III Dextran 150 000

Material and methods

Blood was taken from healthy men and animals either through a cannula in a subcutaneous vein (man horse sheep) in a deep vein (urethane anesthetized rabbit) in an artery (nembutal anesthetized cat, dog and rat) or from the cut vessels of the throat immediately following death by shooting in the slaughter house

The ESR was determined after one hour according to the method of Westergren (1926) Coagulation was prevented either by mixing 4 parts of blood with one part of 0 1 M sodium citrate or by adding 0 1 ml heparin (1 000 I U /ml) per ml blood One ml of this blood was then mixed with 0 4 ml of the test solution if not otherwise is stated

The hematocrit was determined in duplicate by a micro-method using capillary tubes which were centrifuged at 1 500 g for 20 min

In order to study whether an increase in the concentration of certain proteins from human plasma could affect the suspension stability of the blood gammaglobulin or fibrinogen was

TABLE I Mean erythrocyte sedimentation rate of blood from various species without and with concentration 1 7 per cent The molecular weight distribution curve for each dextran

Species	Number of expts	Hematocrit	Controls		
			No addition	Normal saline	5 glucose
Rabbit	4	31 (26 37)	1 (1 1)	1 (1 1)	1 (1 -1)
Rat	3	23 (21 -26)	2 (1 -4)	2 (1--5)	2 (1--4)
Cat	6	30 (2 33)	4 1 10)	2 (1 -3)	2 (1 3)
Swine	4	36 (34- 40	5 (1 12)	3 (1 6)	3 (1--5)
Man	7	33 (31 36	7 2 13	2 (1 -4)	2 (1--3)
Dog	6	31 (24 36	8 (1 2	3 (1 6)	2 (1--3)

added to blood from cow (n = 3) and sheep (n = 2) while albumin was added to blood from horse (n = 6). Gammaglobulin or albumin was added in amounts that increased the blood concentration by 0.85 and 1.7 per cent respectively. Fibrinogen was added to increase the concentration by 0.28 per cent. No determination of the final concentration was performed.

The sedimentation rate of RBC from horse in cow plasma and of RBC from cow in horse plasma was studied by centrifuging a blood sample from one species at 1,200 *g* for 20 min, removing the supernatant and resuspending the RBC in an equal volume of plasma from the other species. In these experiments heparin was used as anti-coagulant.

The following colloids were used:

1. Dextrans obtained by the fermentative action of Leuconostoc mesenteroides strain B 512 and with the average molecular weights ($\bar{M}_w$ light scattering) of about 40 000 (Rheomacrodex®), 75 000 (Macrodex®), 150 000, 250 000 and 500 000 respectively. The distribution curves for the first 3 preparations are given in Fig. 1. All preparations were dissolved in normal saline or 5 per cent dextrose. If not otherwise stated 6 per cent dextran solution was used giving a final dextran concentration of 1.7 per cent in the blood.
2. Albumin, gammaglobulin and fibrinogen were fractionated from human blood with a modification of the method of Cohn. Each preparation was dissolved in normal saline.

Results

A. Addition of dextran

The spontaneous ESR and the effects of normal saline, glucose or equivalent volumes of various dextran preparations are presented in Table I and Fig. 2. There was a great difference in the normal ESR between the species. Cow and sheep had ESR < 1 mm, rat, rabbit, cat, pig, man and dog creating an intermediate group with an average ESR of 1—8 mm, while horse had a mean ESR of 78 mm.

addition of different dextran fractions. Figures within brackets denote range. Final dextran fraction is given in Fig. 1.

Dextran I in		Dextran II in		Dextran III in Normal saline
Normal saline	5 % glucose	Normal saline	5 % glucose	
1 (1—1)	1 (1—1)	16 (2—37)	6 (1—11)	128 (116—136)
1 (0—1)	1 (0—1)	10 (9—12)	4 (3—4)	10[illegible] (42—134)
43 (30—50	8 (4—12)	151 (141—157)	143 (125—154)	—
3 (1—6)	2 (1—3)	64 (28—111)	25 (14—45)	99 (37—136)
2 (1—6	2 (1—4)	78 (35—118)	25 (8—54)	142 (13—147)
2 (1—4	1 (1—2)	64 (12—114)	5 (1—10)	129 (66—150)

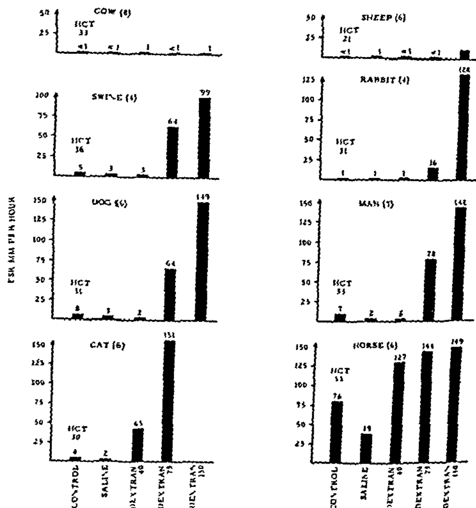


Fig 2 Normal sedimentation rate of RBC (ESR) from various species and the effect of saline and three different dextran preparations on the ESR Final dextran concentration 1 7 per cent

The addition of normal saline of glucose caused a decrease in ESR probably due to a dilution of aggregating proteins in plasma but possibly also to other factors Glucose decreased the ESR more than saline

The effect of the various dextran preparations was different in the various species Dextran with a M_w of 40 000 increased the ESR of cat and horse blood but did not change the ESR in the blood from the other species In all species but cow and sheep dextran with M_w of 75 000 and 150 000 respectively increased the ESR but to various degrees. The suspension stability of cow and sheep blood was not affected by any of the 5 dextran fractions tested

Dextran solutions with glucose generally affected ESR to a lesser degree than those in normal saline. This is in accordance with the finding that 5 per cent dextrose decreased the ESR more than saline (see above).

B. *Addition of proteins*

Fibrinogen or gammaglobulin did not change the ESR of the blood from cow or sheep. The ESR of the horse blood was slightly increased by the addition of albumin (+ 17 %) in comparison with the addition of equal volumes of normal saline, the mean values being 73 mm as compared to 53 mm after saline and 83 mm in the control.

C. *Exchange of plasma*

The initial ESR of cow blood was 0 mm ($n = 2$) and in the horse blood 53 mm and 110 mm respectively. The ESR of cow RBC in the horse plasma became unchanged, *i. e.* zero. The ESR of horse RBC in cow plasma decreased to 32 mm and 38 mm respectively.

Discussion

A big difference in ESR between various species was observed by Hirschfeld (1907) who from experimental evidence concluded that the ESR was a function both of the aggregability of the erythrocytes and of the aggregating potency of the plasma. It is therefore possible that in a given species (*e. g.* man) a high ESR may be caused by changes in the erythrocytes and not only due to a changed plasma protein pattern. This was studied by Fåhraeus (1921) who concluded that in pregnant women the increased ESR was mainly due to plasma factors (for ref. see also Jeannet 1964). Experiments with human erythrocytes suspended in well-defined dextran solution may further elucidate this problem.

In cow and sheep it was not possible to induce sedimentation of the erythrocytes by adding fibrinogen, gammaglobulin or dextran. On the other hand, erythrocytes from these species can be aggregated, and an increased ESR initiated if the erythrocytes are suspended in solution of cellulose derivates, *e. g.* hydroxethylcellulose (Richter, personal communication). The extremely strong aggregating effect of this substance has also been reported by Hint (1962) and Richter (1963).

The post traumatic increase of various macromolecules in plasma most likely plays an important role for the development of intravascular aggregation of red cells ("sludge"). It is not clear if intravascular aggregation occurs in cow or sheep following major trauma. On the other hand, if these animals do not develop "sludge" due to a decrease in the suspension stability of the blood, this will open new ways for studying the controversial question of the relative importance of "sludge" in the complex events of changes that follow for example major traumas.

The mechanism for the increase in ESR in various diseases as well as after the infusion of certain colloids is not known (for review see Illig 1961). It has often been suggested that a change in the electrical charge of the erythrocytes would be the most likely explanation, *i. e.* a decrease in the negative charge would decrease the repelling forces between the red cells. This hypothesis seemed to receive further support from Bernstein *et al.* (1962) who reported that dextran with a M_w of 40 000 caused an increase in the negative charge of the erythrocytes and at the same time caused an increase in their suspension stability. More recent studies revealed, however, that dextran molecules

with strong aggregating potency also caused the same increase in electro-negativity of the red cells (Castaneda *et al* 1964)

The present results indicate that the biological action of dextran is dependent not only on the physico-chemical properties of the dextran preparation but also on the species involved

The authors are indebted to the AB Pharmacia Uppsala for the various dextran fractions and to the AB KABI Stockholm for the plasma proteins

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Adrenergic Transmission at Vasoconstrictor Nerve Terminals Partially Depleted of Noradrenaline[1]

By

GORAN SEDVALL and JAN THORSON

Received 26 October 1964

Abstract

Sedvall G and J Thorson *Ad energic t ansmission at vasoc nst ictor nerve terminals partially deplet d of norad enaline A ta physiol scand* 1965 64 251—258 — The ci culatory responses to acti ation of the vasoconstrictor nerves in skeletal muscle of the cat were studied after partial depletion of the r transmitter stores by the action of reserpine and nerve mpulses In spite of the presence of 30 per cent of the noradrenaline store practically no responses to vasoconstrictor nerve stimulation were obtained provided that the physiological impulse discharge had been impinging on the stores for 2 5 hrs after reserpine administrat on (5 mg/kg i. v.) If the impulse flow had been interrupted during this time 40 per cent of the store was present and normal vasocon strictor responses we e obtained. At 10 hrs after reserp ne only a 10 per cent fraction of the noradrenal ne store was left but normal responses were still produced — provided that no impulses had reached the nerve end ngs after the adm nistration of reserpine About 80 per cent of this small fraction could be depleted by electrical stimulation of the vasoconstrictor nerves No d ff rence in the sensitivity of the blood vessels to injected noradrenaline could be found as a result of decentral zation o d fferences in the durat on of reserp ne treatment. The results suggest that a large part of the norad enaline store in vasoconstrictor nerves is not directly ava lable for release by nerve impulses n the reserp ni ed cat. The ab lity of the nerves to transmit vasoconstrictor impulses appears to be dependent on the presence of a small noradrena l ne fract on constitut ng 10—15 per cent of the total store

As judged from the response to nerve stimulation adrenergic transmission proceeds quite normally even when the transmitter store at the nerve endings is considerably reduced by the action of drugs (Muscholl and Vogt 1958 Rosell and Sedvall 1962 b Gaffney Chidsey and Braunwald 1963 Sedvall and Thorson 1963 Andén Magnusson and Waldeck 1964 Andén 1964) The nerve impulses thus do not seem to release noradrenaline from the store in proportion to its magnitude This is what we would expect if only a small fraction of the transmitter store participates directly in

[1] A p el m nary eport of parts of this study was presented at the Second Pharmacological Meet ng Pragu September 1963

the transmission process a large proportion of the noradrenaline being unavailable for release by nerve impulses (Euler and Lishajko 1961, Trendelenburg 1961, Carlsson 1964). The evidence presented for this view has so far been indirect.

Results were recently obtained indicating that the noradrenaline store of the vasoconstrictor nerves in skeletal muscle is subdivided into at least 2 fractions with different sensitivity to the depletory effect of reserpine (Sedvall 1964 b). The ability of the nerves to transmit vasoconstrictor impulses seemed to be correlated to the presence of a small reserpine resistant noradrenaline fraction rather than to the magnitude of the transmitter store as a whole. The results suggested the possibility of removing the different noradrenaline fractions to some extent selectively (Rosell and Sedvall 1962 a, Sedvall 1964 b).

This possibility was exploited in the present investigation which has aimed at analyzing in more detail the extent to which different noradrenaline fractions in the vasoconstrictor nerves are available for release by nerve impulses. This was accomplished by studying the circulatory responses in skeletal muscle to stimulation of the vasoconstrictor nerves after partial depletion of their transmitter stores by the action of reserpine separately or in combination with nerve impulses.

Methods

The study was made on 37 cats of both sexes, weight 2.0—3.8 kg. The animals were anesthetized with urethane (550—1750 mg/kg i. v.) and the trachea was cannulated. Body temperature was maintained at 36—37 °C by means of a heating lamp. The vasoconstrictor outflow to the hindlimbs of the cat passes through the lumbar sympathetic chains. These were reached by the anterior approach and one or both sympathetic trunks were dissected free and transected at a level of L4 to L5. Reserpine (Serpasil®, Ciba, 5 mg/kg i. v.) was injected and after 2, 5 or 10 hrs the animals were prepared for the recording of blood flow in the muscles of each hindlimb separately. The hindlimb was skinned by diathermia and a tight ligature placed around the ankle. Heparin (25 mg/kg) was injected to prevent clotting. Blood flow in the femoral artery was recorded using the drop chamber technique deviced by Lindgren (1958). Blood pressure was recorded in a carotid artery with a Statham P 23 AA transducer. Responses to vasoconstrictor nerve stimulation and the intra arterial injection of noradrenaline were measured as the increase in peripheral vascular resistance in per cent of the resting level. The peripheral vascular resistance in the hindlimb muscles was calculated as the ratio of pressure to flow. The peripheral ends of one or both transected lumbar sympathetic chains were stimulated at the level of L4—L5 with a bipolar silver electrode. When both sympathetic chains were stimulated simultaneously, they were placed on the same electrode. Monophasic rectangular pulses (5 mS, 5—15 V, 10—20/sec) from a Grass Model S 4 stimulator were used. Atropine (0.5 mg/kg i. v. as sulfate) was given to prevent the action of vasodilator nerve activity. Intra arterial injections of noradrenaline (0.5—5.0 μg) were given via a sidearm of the drop chamber in a volume not exceeding 0.3 ml.

The noradrenaline content of the gastrocnemius muscles was determined in principle as in Bertler, Carlsson and Rosengren (1958). The extracts were purified according to Carlsson and Lindqvist (1962). Oxidation was performed essentially as by Häggendal (1963). For details (recovery etc.) of the methods used see Sedvall (1964 a, b). The noradrenaline content has been expressed as free base in ng/g wet weight of muscle.

Results

The results of Rosell and Sedvall (1962 a) and Sedvall (1964 b) suggested that the physiological impulse flow depletes a functionally important noradrenaline fraction in the vasoconstrictor nerves of skeletal muscle within a few hours after reserpine administration. This possibility was further examined in a first series of experiments. Immediately

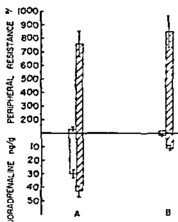


Fig 1 Noradrenaline content and peripheral vascular resistance in cat skeletal muscle following sympathetic chain stimulation 2 5 (A) and 10 hrs (B) after reserpine administration (5 mg/kg i v)
Shaded columns indicate that the sympathetic chain was transected prior to reserpine administration
Plain columns signify that the sympathetic chain was transected 5 min before stimulation
Each column is the mean of 5 expts Vertical bars indicate ± S E For full explanation see text

before the administration of reserpine (5 mg/kg i v) the flow of vasoconstrictor impulses to one hindlimb was interrupted by transecting the ipsilateral lumbar sympathetic chain The physiological impulse discharge in the vasoconstrictor nerves to the other hindlimb was allowed to continue for about 2 5 hrs after reserpine injection. The sympathetic chain also on that side was then transected A time interval of at least 5 min was allowed in order to rest the effector cells from any transmitter action after which the peripheral ends of the two sympathetic chains were simultaneously stimulated electrically and the vasoconstrictor responses registered in the hindlimb muscles Fig 1 A presents the results from 5 cats in which practically no response at all was obtained on the side where the impulse flow had passed uninterrupted during about 2 5 hrs of reserpine action There was still about 30 per cent of the normal noradrenaline content left in the muscles on this side The content of noradrenaline in the muscles on the other side, where the vasoconstrictor nerves had been decentralized before reserpine was administered was only slightly higher but the vasoconstrictor response was normal The difference in the amount of noradrenaline — which was accompanied by such an important difference in response to nerve stimulation — was only 10—15 per cent of the normal content. Similar results were obtained if the effector cells were rested for about 30 min before stimulation

In another series of 5 cats the time interval between reserpine administration and transection of the second sympathetic chain was 10 hrs instead of 2 5 hrs Practically identical results were obtained as regards the vasoconstrictor responses, but onl about 10 per cent of the normal noradrenaline content was left on the decentralized side and only about 2 per cent on the side where the nerve impulses had passed uninterrupted (Fig 1 B) The noradrenaline content of normal skeletal muscle is about 100 ng/g

The differences in vasoconstrictor responses obtained in the above experiments might have been due to a changed sensitivity of the effector cells to the released transmitter This possibility is rendered highly improbable by the results of the 2 following series of experiments These were identical to those described above except that instead of stimulating the vasoconstrictor nerves we injected noradrenaline i a into both hind

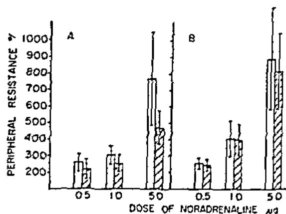


Fig 2 Peripheral vascular resistance in cat skeletal muscle following intra arterial injection of noradrenaline 2.5 (A) and 10 hrs (B) after reserpine administration (5 mg/kg i v)
Shaded columns indicate that the lumbar sympathetic chain was transected before reserpine administration
Plain columns signify that the sympathetic chain was transected 5 min before noradrenaline injection
Each column is the mean of 4 expts Vertical bars represent ± S E *For full explanation see text*

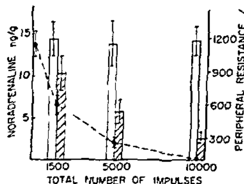


Fig 3 Noradrenaline content and peripheral vascular resistance in cat skeletal muscle following sympathetic chain stimulation after 10 hrs reserpine treatment (5 mg/kg i v)
Both sympathetic chains were transected immediately before reserpine injection
Shaded columns noradrenaline content stimulated side
Plain columns noradrenaline content, non stimulated side
Dotted line vasoconstrictor response to a defined test stimulus (300 imp 10 sec) stimulated side
Each point and column is the mean of 5 expts Vertical bars indicate ± S E For full explanation see text

limbs The data in Fig 2 clearly show that no significant change in sensitivity to injected noradrenaline occurred as the result of decentralization or the difference in the duration of reserpine treatment

A series of experiments was deviced to investigate how far the noradrenaline fraction remaining in decentralized vasoconstrictor nerves 10 hrs after reserpine was available for release by nerve impulses Both sympathetic chains were transected before the administration of reserpine After an interval of about 9 hrs when about 10—15 per cent of the normal noradrenaline content is still present (Fig 1) one of the *sympathetic chains was stimulated electrically with a certain number of* stimuli The vasoconstrictor response to a defined test stimulus (300 imp 10 sec) was measured following the delivery of 300 1 500 5 000 and 10 000 stimuli Three series of 5 cats were killed after delivery of 1 500 5 000 and 10 000 stimuli respectively and the noradrenaline content in both gastrocnemius muscles determined The results can be seen in Fig 3 A definite relationship was obtained between the drop in noradrenaline content and the total number of stimuli Following the delivery of 10 000 imp the noradrenaline content (3 ± 0.8 ng g mean ± S E n = 5) was highly significantly (p < 0.001) reduced in relation to the nonstimulated control side (14 ± 1.8) The *fraction* was

reduced by about 80 per cent i e to the same level as obtained when a physiological impulse flow had passed for 10 hrs (Fig 1) A similar relationship was obtained between the total number of stimuli and the reduction in vasoconstrictor responses There was always a continuous reduction in blood flow through the muscles in these animals during the stimulation period due to the bad circulatory condition of the cat when treated for several hours with high doses of reserpine (Withrington and Zaimis 1961) The vasoconstrictions obtained in the late part of the stimulation period may thus have been slightly too small owing to a changed pressure flow relationship (Renkin and Rosell 1962 Lewis and Mellander 1962) However this circumstance cannot have altered the general slope of the curve as practically no vasoconstrictor response was obtained following the delivery of 10 000 imp

Discussion

The noradrenaline content of cat skeletal muscle is localized almost exclusively in the vasoconstrictor nerves (Sedvall 1964 a Fuxe and Sedvall 1964 1965) The present results showed that vasoconstrictor nerve function was lost before total depletion of the transmitter stores after reserpine In spite of the presence of 30 per cent of the noradrenaline practically no responses to vasoconstrictor nerve stimulation were obtained when the physiological impulse discharge had been impinging on the stores for 2 5 hrs after reserpine administration (Fig 1) Three different explanations seem possible for this failing response (1) a lack of available transmitter substance in the nerve endings (2) an impaired release mechanism for the stored transmitter and/or (3) failing reactivity of the effector cells This last explanation seems highly improbable as the blood vessels showed the same sensitivity to injected noradrenaline as those on the control side where a normal response to nerve stimulation was obtained The same applies to the second possibility It is unlikely that reserpine *per se* blocked the release mechanism as the latter functioned normally on the control side The explanation that the physiological impulse flow — which was presumably rather high (Iggo and Vogt 1960) — exhausted some factor responsible for the release of transmitter is also unlikely Under normal conditions even prolonged adrenergic nerve stimulation with supra physiological frequences does not lead to fatigue of the transmission (Orias 1932 Dye 1935 Kernell and Sedvall 1964) The most reasonable explanation is that the noradrenaline fraction which was present when transmission failed is stored unavailable for release by nerve impulses

There is a possibility that the unavailable fraction is stored in the preterminal portions of the axons It has been shown however that reserpine depletes the noradrenaline stores in different parts of the adrenergic neurone at about the same rate (Norberg and Hamberger 1964 Dahlstrom and Fuxe 1964) If the entire 30 per cent fraction was present in the preterminals the amount of noradrenaline normally kept in this part of the neurone must be considerably larger This seems incompatible with the recent histochemical findings of Malmfors (1964) which strongly indicate that the noradrenaline in rat iris is stored predominantly in true adrenergic terminals readily available for the action of nerve impulses It therefore seems probable that at least a large part of the unavailable fraction is present in the true terminals but still not accessible for release by nerve impulses The fact that this transmitter fraction was not rapidly made available could indicate that mobilization of the transmitter is normally very slow However it is also possible that reserpine blocks one of the processes which make the

preformed transmitter accessible for release by nerve impulses The findings of Euler and Lishajko (1961) and Johnson (1964) are of interest in this connection These authors presented evidence from *in vitro* and *in vivo* studies indicating that reserpine in small doses blocks the release of noradrenaline from storage granules

The present results thus suggest that a large part of the noradrenaline in the vasoconstrictor nerves is stored unavailable for release by nerve impulses after reserpine treatment To what extent then is the transmitter available?

Considerable evidence indicates that reserpine blocks an uptake mechanism for amines in the catecholamine storing granules (Carlsson Hillarp and Waldeck 1962 1963 Kirshner 1962 a b Euler and Lishajko 1963 Hillarp and Malmfors 1964) There is a close time correlation between the inhibition of this uptake and the impairment of adrenergic nerve function in the recovery period following reserpine treatment (Lundborg 1963 Carlsson Jonasson and Rosengren 1963 Andén Magnusson and Waldeck 1964 Stjarne 1964) This indicates that the uptake of amines into granules is of great importance in the mechanism that makes the transmitter available for release (Carlsson 1964) No transmitter was presumably made available by uptake into granules in the present experiments which were performed within a few hours after reserpine administration Moreover the present results suggest that noradrenaline was not mobilized from the unavailable store In these circumstances the ability of the nerves to release transmitter should be dependent mainly on the amount of preformed noradrenaline which is actually stored available A correlation existed between the ability of the nerves to transmit impulses and the 10—15 per cent fraction of the normal amount (Fig 1 and 3) Moreover this fraction could be rapidly depleted by 80 per cent by stimulation of the vasoconstrictor nerves These findings suggest that in the reserpinized animal only this fraction is immediately available for release by nerve impulses This infers that the noradrenaline in the vasoconstrictor nerves is retained in at least 2 pools

It was recently shown that decentralization of the vasoconstrictor nerves in skeletal muscle disclosed a small noradrenaline fraction in the nerve terminals which disappeared at a slower rate than the main part of the transmitter store after reserpine (Sedvall 1964 b) The ability of the vasoconstrictor nerves to transmit impulses as found by Rosell and Sedvall (1962 a) appeared to be dependent on the presence of this fraction The present study supports the view that the available fraction demonstrated here is identical with this slowly disappearing fraction The results in Fig 1 and 3 thus indicate that practically only the available fraction remained at 10 hrs after reserpine — provided that no impulses had passed Under almost identical conditions it was found that predominantly the reserpine resistant fraction was left at this interval (Sedvall 1964 b) The evidence accordingly indicates that the available noradrenaline fraction in the vasoconstrictor nerves differs from the main part of the store not only in being more directly accessible for release by the nerve impulses but also in disappearance — in the absence of impulse discharge — at a slower rate after reserpine administration These findings strongly indicate that this fraction is stored in the nerve terminal in a special compartment Future experiments must decide whether this subdivision of the transmitter store in the vasoconstrictor nerves is due to the existence of different storage mechanisms for the transmitter and/or to differences in the location of the stored transmitter in relation to the axon membrane

Quantitatively the nerve impulse discharge plays only a minor role in catecholamine depletion after reserpine (see Sedvall 1964 b) The present results however directly

show that the physiological nerve impulse flow plays an important role — from a functional point of view — in the mechanism of action of reserpine It seems to be responsible for the rate of depletion of the small noradrenaline fraction which mediates the transmission of impulses The rate of disappearance of adrenergic nerve function after reserpine will accordingly be highly dependent on the impulse frequency in the nerves The results presented provide a reasonable explanation for the general lack of correlation between amine levels and adrenergic nerve function following reserpine treatment and for the finding that adrenergic transmission after reserpine can be abolished before total depletion of the transmitter stores (Sedvall and Thorson 1963 Haggendal and Lindqvist 1964)

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Acta physiol scand 1965 64 259—262

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Absence of Monoamines in Olivo-Cochlear Fibres in Cat

By

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Received 26 October 1964

Abstract

Fex J K Fuxe and G Lennerstrand *Absence of monoamines in olivo-cochlear fibres in cat* Acta physiol scand 1965 *64* 259—262 — The olivo-cochlear neurons in the cat have been studied with the help of the fluorescence method of Falck and Hillarp with or without crushing of their axons in the vestibulo-cochlear anastomosis or in the pons No monoamines were found in these neurons (cell bodies axons terminals) not even after axotomy It is concluded that primary catecholamines and 5 hydroxytryptamine in all probability do not act as transmitters in the efferent innervation of the organ of Corti

The course of the crossed olivo-cochlear efferent fibres was first described by Rasmussen (1946) and this has been followed by several other studies (see Rasmussen 1960 Rossi and Cortesina 1962) The uncrossed olivo-cochlear efferents were also found by Rasmussen (1960) and again described by Rossi and Cortesina (1962)

Electronmicroscopical investigations on the cochlea after transection of the crossed or both the crossed and uncrossed cochlear efferents (for references see Spoendlin and Gacek 1963) prove that the crossed efferents terminate on the hair cells and the afferent endings of the organ of Corti The uncrossed efferents probably end in the same region (*cf* Spoendlin and Gacek 1963)

Electrophysiological studies have shown the crossed efferents to be inhibitory in function (for references see Fex 1962)) as are also the uncrossed (Desmedt and LaGrutta 1963)

The cochlear efferents have a high acetylcholinesterase (AChE) activity (for references see Rossi and Cortesina 1962) and they may thus be cholinergic releasing acetyl choline (ACh) as a transmitter In support of this view Gisselsson (1960) found an increase in the cochlear microphonics after injection of ACh into the endolymph and related these findings to the earlier results that the cochlear microphonics are increased

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by electrical stimulation of the crossed efferents (Fex 1959). The effect of such electrical stimulation has since been studied also by Desmedt and co-workers (see Desmedt 1962). However, electrophoretic application of acetylcholine into the organ of Corti as done by Katsuki (see Bullock 1964) caused suppression of the cochlear microphonics although a full report of these experiments has still to appear. Furthermore, it has been shown (Desmedt and LaGrutta 1963) that the effect of cochlear efferents on the cochlear action potential and microphonics are not changed by i. v. injected physostigmine (an AChE-inhibitor) nor by dihydro-β-erythroidine which blocks transmission in cholinergic junctions (Unna, Kniazuk and Greslin 1944).

The non cholinergic nature of the efferent transmitter is also suggested by the findings that subconvulsive doses of strychnine reduced or abolished the effects of electrical stimulation of the olivo-cochlear fibres (for references see Fex 1962 and Desmedt and LaGrutta 1963). The cochlear efferents are in this respect similar to the spinal interneurons that are responsible for post synaptic inhibition of motoneurones and that are in all probability non cholinergic (*cf* Curtis 1963). However, the crossed efferent inhibition is not affected by 5,7 diphenyl 1,3-diazadamantan 6-ol (1757 I.S.) which depresses spinal post synaptic inhibitions (*cf* Curtis 1963). Thus these spinal and the cochlear inhibitory systems may have different inhibitory transmitter substances.

It has been found that the terminals of many different neuron systems in the CNS form and store monoamines (see Carlsson, Falck and Hillarp 1962, Dahlström and Fuxe 1964a). These neurons have recently been shown to be monoaminergic (Fuxe and Gunne 1964). The present work was made to establish whether the cochlear efferents belong to any of these specific systems. The presence and direction of monoaminergic fibres can now be directly studied in the central nervous system with the use of a simple method (Dahlström and Fuxe 1964b). The method is based on the finding that after axotomy the respective amines rapidly accumulate in high amounts readily visualized with the fluorescence method of Falck and Hillarp in the proximal part of the fibres.

Material and methods

10 cats free from ear infections and weighing between 1.5 and 4.0 kg were used in the present study. In 5 anesthesized cats attempts were made to take out pieces of the organ of Corti keeping the topographical interrelationships intact. However, the organ was regularly damaged and the specimens contaminated with bone tissue.

In the 5 remaining cats (intraperitoneal pentobarbital 35 mg/kg) the left vestibulo-cochlear anastomosis, containing the cochlear efferents, was crushed with a fine forceps under aseptic conditions. The technique to reach Oort's anastomosis has been described by Fex (1962). In 2 of the cats the crossed cochlear efferents (see Rasmussen 1946) were interrupted also in the brain stem when crossing at the midline just under the floor of the fourth ventricle at the level of the genu of the facial nerves (see Fex 1962). After the operation the animals were kept alive for 20 hrs under light pentobarbital anesthesia with control of airways and body temperature and then killed by additional pentobarbital. The stato-acoustic nerves on both sides were taken out from the cats in which the left vestibulo-cochlear anastomosis had been crushed. Specimens from around the cut in the brain stem were taken in the 2 cats with such a lesion and control specimens from the same regions were taken in the cats which had the brain stems intact. In all cats the regions which would contain the cells of origin of the cochlear efferents (*cf* Rasmussen 1946, Ross and Cortesina 1962) were taken as specimens.

All specimens were freeze-dried, treated with formaldehyde gas and embedded in paraffin as described previously (Dahlström and Fuxe 1964a). The serial sections (8–10 μ) were examined by fluorescence microscopy. The nerves were cut longitudinally and the brain specimens transversely.

Under the conditions of the histochemical reaction primary catecholamines, such as dopamine (DA) and noradrenaline (NA) and 5-hydroxytryptamine (5-HT) are transformed into intensely fluorescent 6 7-dihydroxy 3 4-dihydro-isoquinol nes and 6-hydroxy 3 4-dihydro-β-carboline respectively (Corrodi and Hillarp 1963 1964) Thanks to this conversion the presence of 5-HT and catecholamines is visualized by what in the fluorescence microscope used is a yellow and usually green to yellowgreen fluorescence respecti ely

Results

Normal animals

The organ of Corti was severely damaged in all preparations No nerve fibres with specific fluorescence were present around the few sensory cells that could be observed The stato-acoustic nerves contained a number of fine varicose green fluorescent nerve fibres some of which seemed to be related to blood vessels They were running in a part of the nerve where there are no olivo-cochlear fibres The superior olivary complex and its immediate surroundings which would include the cells of origin of all olivo-cochlear fibres (*cf* Rasmussen 1946 1960 Rossi and Cortesina 1962) did not contain any nerve cells with catecholamines or 5-HT

Animals with the left vestibulo-cochlear anastomosis crushed

No accumulation of catecholamines or 5-HT could be observed in the fibres above or below the lesion

Animals with section of the crossed olivo-cochlear fibres in the brain stem

A number of intensely green and yellow fluorescent deformed nerve fibres were observed on each side of the lesion These fibres however had a location and a direction different from those of the cochlear efferents (as described by Rasmussen 1946)

Discussion

The method used here to find monoamines in neurons is highly sensitive and specific (for references see Dahlstrom and Fuxe 1964a) Furthermore axotomy of monoamine containing nerves fibres results in a rapid and large accumulation of monoamines in the proximal part of the fibre in the central as well as in the peripheral nervous system (Dahlström and Fuxe 1964b)

In the present work no monoamines were found in the region of the hair cells and in spite of the axotomies performed no monoamines were found in the axons or in the soma of the cochlear efferent neurons However certain parts of the vestibular ganglion have been found to contain CA terminals in close contact with the ganglion cells (Dahlström Fex Fuxe and Lennerstrand unpublished observations) Experiments are in progress to show if they are of central or peripheral origin

It is concluded that olivo-cochlear efferent fibres do not contain NA DA or 5-HT hence it is unlikely that these monoamines are transmitter substances at the termination of these efferents upon the cells of the organ of Corti

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Acta physiol scand 1965 64 263—268

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Exchangeable Water, Sodium and Chloride in the Skin of Mice

By

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Abstract

Langgård H *Exchangeable water sodium and chloride in the skin of mice* Acta physiol scand 1965 64 263—268 — The exchangeability of water sodium and chloride in skin has been studied in untreated mice in mice with localized acute inflammatory edema and in mice with estradiol induced edema of the skin The total amounts of water and sodium were found to be readily exchangeable whereas 15 per cent of the tissue chloride in untreated mice was exchanged at a much lower rate The findings in the mice with acute inflammatory edema and in mice treated with estradiol indicated a possible relationship between the slowly exchangeable chloride and the amount of acid mucopolysaccharides in the skin

Previous studies on the composition of connective tissue have indicated different fluid phases or "water compartments" Terms like "bound" or "fixed" water "free water" and "freely moveable" water have often been applied to some of these fractions without precise definitions The various fractions of tissue water may contain electrolytes in different concentrations Electrolytes may further be stored in the tissue in a "dry" or "osmotic inactive" form "attached" to or "bound" to various charged macromolecules

Thus for the biological function the total contents of water and electrolytes is of less interest than the part "available" to the organism in the homeostasis The available (exchangeable) part can be determined by isotope dilution methods

The purpose of the present paper has been to study the exchangeability of water sodium and chloride in connective tissue with special regard to the function of the acid mucopolysaccharides of the ground substance

Methods

White male mice of a single strain weighing from 22 to 28 g were used They were maintained on a standard laboratory diet with water ad libitum

Experimental groups

1) *Mice with estradiol induced edema of the skin*

On the 6th and 4th day before the experiments subcutaneous injections of estradiol monobenzoate 10 γ g in 0 1 ml of arachis oil were given (cf Hvidberg Szporny and Langgård 1964)

2) *Mice with localized acute inflammatory edema*

On the day prior to the experiment the skin on the back was depilated by a close cut with an electric clipper followed by application of a barium sulphide depilatory After 5 min the skin was cleared of depilatory with ample amounts of water One hour before the experiment the animal was lightly anesthetized with fluothane (halothane® B P) and acute inflammatory edema induced on the naked skin by applying filter paper moistened with xylene for 2 min (cf Szporny *et al* 1964)

3) *Control animals* (untreated)

Experimental procedures

A) *Exchangeable water*

Using a micrometer syringe (Agla®) 50 γ l of a solution of ^3H_2O in 0 9 per cent NaCl containing approximately 1 μC was injected into a tail vein and the site of puncture sealed carefully Ten minutes later the animal was stunned by a neckblow the right carotid artery was cut and 500 γ l of blood sampled in a Carlsberg constriction pipette prepared with heparine saline After decapitation and bleeding the backskin was depilated as described above (animals with localized acute edema were already depilated at this stage) A particular area of the depilated skin with underlying subcutaneous tissue was excised and divided into two pieces which were weighed on a torsion balance One sample was used for determination of the water content by freeze-drying of the sample to constant weight The other sample was used for the radioactive assay N/2 sodium hydroxide in a volume of 3 times the tissue volume (spec grav = 1 0) was added and the sample heated in a stoppered tube to 70 C for 30 min to obtain tissue destruction The proteins were precipitated by adding 10 zinc sulphate in the same volumes as sodium hydroxide Plasma samples were treated in an identical way After centrifugation 100 μl of the clear supernatants were added to a scintillation medium described by Bray (1960) and the radioactivity measured in a liquid scintillation counter (Isotope Developments LTD) The extent of penetration of ^3H_2O into the tissue was determined by comparing the ratio of the tissue plasma radioactivity to a similar ratio for water determined chemically by freeze-drying of the tissue When the two ratios were equal penetration was complete

B) *Exchangeable sodium*

Approximately 1 γ C of ^{22}Na as NaCl in 50 μl of a 0 9 per cent NaCl solution was injected i v Ten minutes later blood was sampled and the backskin depilated and excised as described for determination of exchangeable water The total skin sample was weighed on a torsion balance put into counting tubes and the radiation measured in a well type crystal connected with a 1700 scaler (Isotope Developments LTD) The radioactivity of 50 μl of blood plasma was determined simultaneously After the radioactive assay the skin samples were freeze-dried to constant weight and defattened by repeated treatments with petroleum ether and di-ethyl ether The dry fatfree tissue and the plasma were finally analyzed for sodium with a Beckman DU flame photometer and photomultiplier (cf Hvidberg Jensen Holm and Langgård 1963) The ratio of the tissue plasma concentrations of ^{22}Na and the same ratio for Na determined chemically were compared If penetration of ^{22}Na into the tissue was complete the two ratios should be equal

C) *Exchangeable chloride*

50 γ l of a 0 9 per cent NaCl solution containing approximately 1 μC of ^{36}Cl as NaCl was injected i v Groups of animals were examined 10 min 2 hrs or 20 hrs later Otherwise the procedures were the same as described for determination of exchangeable water The total

TABLE I The percentage of water, sodium and chloride which is exchanged in 10 minutes

	Normal skin	Skin of estradiol treated mice	Skin with acute inflammatory edema
H_2O	101±3.9 (n=11)	97±3.1 (n=12)	100±2.6 (n=11)
Na	105±2.7 (n=10)	102±4.3 (n=10)	103±3.2 (n=10)
Cl	85±1.9 (n=12)	71±2.7 (n=12)	91±1.6[1] (n=10)

The values are *means* ± standard *error*
n = number of animals in each group
[1] Significantly different from 100 at $p < 0.001$
Significantly different from the corresponding control group at $p < 0.001$

amounts of chloride in the dry fat free tissue and in the plasma were determined by automatic potentiometric titration with silver nitrate (Radiometer's titrator) (*cf* Langgård, Jensen, Holm and Hvidberg 1963), in the tissue after precipitation of the proteins by Somogyi's technique (*cf* Cotlove 1963). The tissue : plasma ratios of radioactivity and total chloride determined chemically were compared. The percentage recovery of radioactivity from skin and plasma samples were determined by adding known amounts of Cl to skin and plasma samples. No statistically significant difference was found between the efficiency of measuring Cl added to 10 skin samples and 10 plasma samples ($p > 0.1$ by t test).

Results

The exchangeable part (E) of water, sodium and chloride was calculated by the formula

$$E \text{ (per cent)} = \frac{\dfrac{\text{counts per sec per mg tissue}}{\text{counts per sec per mg plasma}}}{\dfrac{\text{total content per mg tissue}}{\text{total content per mg plasma}}} \times 100$$

The values obtained 10 min after administration of the radioactive material are shown in Table I. The figures indicate that the exchange of water and sodium was completed at this time. Only 85 per cent of the tissue chloride were, however, exchanged in untreated animals. In estradiol treated animals the figure was as low as 71 per cent but 91 per cent in animals with localized acute inflammatory edema.

The same ratio was therefore determined in normal and estradiol treated animals 2 and 20 hrs after injection of the radioactive chloride. The findings are illustrated in Fig. 1. Two hours after the injection 95 per cent (S.E. = 3.7) were exchanged in untreated mice, 82 per cent (S.E. = 2.0) in estradiol treated animals. At 20 hrs the exchange was complete in normal skin (102 per cent ± 3.5) but not in skin of estradiol treated mice (97 per cent ± 3.0).

Discussion

The water of the skin is distributed between the intracellular and the extracellular space. The extracellular space in turn consists of an intravascular compartment and several interstitial fluid compartments. The water of these compartments is held by

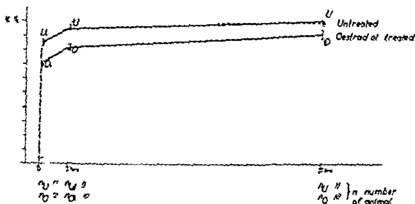


Fig 1 Exchanged chloride in per cent of the chloride content determined chemically 10 min 2 hrs and 20 hrs after injection of radioactive chloride The values for the standard error of the mean are indicated by vertical lines through the points Point O is significantly different from O U from U O from O O from U O from U_0 and O from 100 per cent at $p < 0.01$

forces of different natures and strength If skin is exposed to a rising mechanical pressure a certain fraction of the water can be squeezed out (Hvidberg 1959) The remaining part is held so tightly that it appears to be bound in the tissue Eilers and Labout (1946) stated that approximately half of the bound water does not serve as solvent for small ions (non electrolyte dissolving water) This part of the tissue water may be bound to tissue proteins in a crystallized form by electrostatic forces generated by hydrophilic non polar sidegroups of the proteins on the dipoles of water (Fraser and McRae 1959 Klotz 1960) The other half which is somewhat less adherent was thought to be trapped within minute tissue cavities of the colloidal matrix (nonsaccharose dissolving water) However the water binding capacity of connective tissue is predominantly related to the hyaluronic acid (cf Hvidberg 1960) Kulonen (1952) points out that hyaluronic acid binds water in two essentially different ways Large amounts of water can be taken up due to the gel structure In addition small ions attached to the hyaluronic acid to the extent that they are osmotic active will attract water from the surrounding fluid Consequently another phase of fixed water adhering to the molecules of the hyaluronic acid must be counted for A freely movable fluid phase may also exist in connective tissue (McMaster and Parsons 1957)

The data of the present study (Table I) indicate that no difference exists between the exchangeability of the water molecules in these different compartments Any water molecule in the connective tissue regardless of which compartment it belongs to regardless of which component it is bound to and regardless of the nature of the binding forces is as readily exchangeable as any other water molecule of the tissue In other words bound water in the sence of less exchangeable water molecules does not exist in connective tissue The exchange of tritium does not determine the water phase only but also exchangeable hydrogen ions in proteins hyaluronic acid etc The dissociation of water at tissue pH is however inconsiderable and the exchange of hydrogen ions would therefore not influence the radioactive assay of tritiated water to a measureable extent

Similar statements can be made with regard to sodium (Table I) Calculations on the osmolarity of the tissue fluid shows that a great fraction of the cations must be present in such a way that the osmotic activity is appreciably reduced (Hvidberg Jensen Holm and Langgård 1963 Langgård *et al* 1963) The hyaluronic acid seems to be responsible for the binding of excess sodium and potassium (Kulonen 1952 Hvidberg Szporny and Langgård 1964) It is not evident whether collagen also binds sodium Kahn *et al* (1962) studying the effects of electrolytes on collagen in solution showed evidence of ion binding They further stated that it was difficult to remove ions bound to collagen although some exchange of ions took place According to Harris and Steinbach (1956) there is within isolated frog muscle a small fraction of sodium which is practically non exchangeable This non-exchangeable sodium seemed to be associated with the connective tissue rather than with the muscle fibres Manery and Bale (1941) however studying the penetration of radiosodium into the extra and intracellular phases of the tissues of rats found rapid exchange in skin kidney liver and muscle The same authors stated that there is an essential difference between the availability of the excess sodium of connective tissue and bone In contrast with the excess sodium of bone that of connective tissue is freely exchanged with sodium in plasma

In agreement with the last mentioned authors the present study indicates that there is free exchange of sodium ions between the different compartments of the connective tissue and sodium ions in plasma

For a long time there has been a standing discussion whether chloride ions can be stored dry in the skin It is generally accepted now that the colloids of the connective tissue ground substance have amphoteric properties resulting from the coexistence of positively and negatively charged groups (Ussing *et al* 1960) Thus anions can be bound as well as cations although at physiological pH connective tissue is characterized by a net negative charge Engel *et al* (1961) in in vivo experiments convincingly demonstrated the anion binding capacity of dermis by a potentiometric method Manery and Haege (1941) using Cl with a short life time studied the extent and rate of penetration of chloride in different tissues They found that penetration was completed in skin within a few minutes In contrast with this the data of the present study strongly suggest that a certain fraction of the tissue chloride is exchanged at a much lower rate than is the main part (Table I) In skin of untreated mice this part constitutes approximately 15 per cent of the total chloride content The findings in animals pretreated with estradiol and in animals with localized acute inflammatory edema indicate that the slowly exchangeable chloride may in some way be related to the amount of acid mucopolysaccharides of the skin Treatment with estradiol in this specific strain of mice raises the amount of acid mucopolysaccharides in the skin approximately 100 per cent without affecting the collagen content (*cf* Hvidberg Szporny and Langgård 1964) On the supposition that the slowly exchangeable chloride fraction is related to the amount of acid mucopolysaccharides treatment with estradiol should raise the percentage of slowly exchangeable chloride from the above 15 per cent to approximately 30 per cent which as actually found (Table I) Skin samples with localized acute inflammatory edema per unit surface area weigh approximately the double of non inflamed skin samples (*cf* Langgård *et al* 1964) and the relative amount of acid mucopolysaccharides is therefore decreased to approximately 50 per cent of the normal Thus the percentage of slowl exchangeable chloride accord ng to

the same hypothesis should go down from the above 15 per cent to approximately 7 5 per cent which was also found (Table I)

Of course no conclusive statement as to the nature of the slowly exchangeable chloride ions can be made on basis of the data presented here The data obtained 2 and 20 hrs after injection of the radioactive chloride (Fig 1) suggest that the slowly exchangeable chloride is exchanged at a rather constant rate

This investigation was carried out in The Laboratory for Investigation of the Pharmacology of the Connective Tissue with a grant from the Danish National Association against Rheumatic Diseases

The author thanks Miss Lene Clausen for exellent technical assistance

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Acta physiol scand 1965 64 269—274

From The Department of Physiological Chemistry University of Lund Sweden

The Transport Form of Free Fatty Acids in Rat Serum

A study in vitro using gel filtration

By

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Abstract

Goransson G *The transport form of free fatty acids in rat serum A study in vitro using gel filtration* Acta physiol. scand 1965 64 269—274 — The partition of labeled fatty acids between the protein and water phases in rat serum has been studied in vitro using gel filtration on Sephadex G-200 columns Lauric capric and caproic acids were to an increasing degree distributed over the total volume of the column whereas all saturated fatty acids as well as unsaturated acids with a chain length of 14 carbon atoms or more were completely eluted in combination with protein The saturated acids were with increasing number of carbon atoms to an increasing extent eluted with the lipoprotein fraction More than 98 of all the unsaturated fatty acids studied were eluted with the albumin peak. When the amount of oleic acid was increased above a mole ratio of total fatty acid to albumin of 3—6 the percentage of radioactivity recovered with the albumin fraction decreased

The interaction of free fatty acid anions with albumin has been demonstrated by several authors (Teresi and Luck 1952 Goodman 1958) Using electrophoresis Laurell (1955) and Gordon (1955) have shown an interaction also between oleate and serum lipoproteins

In the present work the partition of labeled fatty acids between the serum protein and the water phases has been studied in gel filtration experiments

Materials and methods

The fatty acids used were the following

Caproic acid 1 C	Obtained from The Radiochemical Centre Amersham England. Batch 4 Spec act 4 mc mM
Capric acid 1 C	Obtained from The Radiochemical Centre Amersham, England Batch 9 Spec act. 4 mc mM

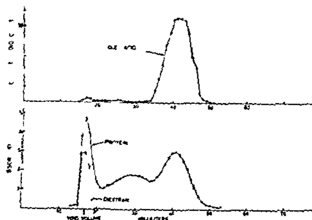


Fig 1 C¹⁴-oleic acid complexed with rat serum subjected to gel filtration using Sephadex G 200 column chromatography Gel bed 60 ml Void volume 17 ml determined with dextran (m w 500 000)

Lauric acid 1 C¹⁴	Obtained from The Radiochemical Centre Amersham England Batch 9 Spec act 21 mc/mM
Myristic acid H	Nonlabeled acid obtained from the Hormel Institute Austin Minnesota USA The acid was labeled with H as described by Bergstrom and Lindstedt (1957) Spec act 10 mc/mM
Palmitic acid 1 C	Obtained from The Radiochemical Centre Amersham England Batch 22 Spec act 4 mc/mM
Palmitic acid 9 10-H	Obtained from The Radiochemical Centre Amersham England Batch 3 Spec act 390 mc/mM
Palmitoleic acid H	Prepared biosynthetically from palmitic acid 9 10 H (see above) as described by Goransson (1965 c) Spec act 7 mc/mM
Stearic acid 9 10-H	Obtained from The Radiochemical Centre Amersham England Spec act app 100 mc/mM
Oleic acid 1-C	Obtained from The Radiochemical Centre Amersham England Batch 1 Spec act 25 mc/mM
Linoleic acid 1 C	Obtained from The Radiochemical Centre Amersham England Batch 20 Spec act 25 mc mM
Arachidic acid 1 C	Obtained from Calbiochem California USA Spec act ? mc mM
Oleic acid unlabeled	Obtained from Fluka AG Basel Switzerland Was used in the chemical amounts of 0 01 0 13 2 7 and 10 µeq per ml of serum in combination with labeled oleic acid to obtain an increasing molar ratio fatty acid albumin in four solutions

Each fatty acid except caproic capric and linoleic was subjected to reversed phase chromatography and immediately before the preparation of an injection solution liquid liquid partition as described by Borgstrom (1952) to assure that more than 99 5 of the radioactivity was present as the respective free fatty acid No attempt was made to purify the caproic and capric acid The linoleic acid was purified by reversed phase column chromatography and AgNO SiO thin layer chromatography as described by Goransson (1965 e) and the radiochemical purity was approximately 99 5

The amount of the sodium salt of each fatty acid given in table 1 was dissolved in 0 ? ml of ethanol and 1 ml of freshly prepared serum from normal rats added The mixture was allowed to stand at least five hours at room temperature and was then filtered The serum was usually clear (except when 10 µeq of Na-oleate was dissolved in 1 ml of serum) Each solution contained palmitic acid and another fatty acid so that one C and one H labeled fatty acid were present in each solution

TABLE I The partition of labeled fatty acids between protein and water phases in rat serum studied by gel filtration on Sephadex G 200 columns Gel bed 60 ml Void volume 17 ml

		Percentage of radioactivity eluted with		
	μEq of fatty acid added to 1 ml of serum	13—20 ml of eluent	35—50 ml of eluent	50—70 ml of eluent
Caproic acid	0.02	0	50	50
Capric acid	0.04	1	93	6
Lauric acid	0.05	2	96	2
Myristic acid	0.05	2	98	0
Palmitic acid	0.04	4±0.8	96±0.8	0
Stearic acid	0.05	19	81	0
Arachidic acid	0.06	71	29	0
Palmitoleic acid	0.20	2	98	0
Linoleic acid	0.08	2	98	0
Oleic acid	0.01	1	99	0
Oleic acid	0.13	1	99	0
Oleic acid	2.7	30	70	0
Oleic acid	10.0	55	45	0

To each solution was also added 0.04 μEq of palmitic acid so that each solution contained one C^{14} labeled and one H^3 labeled fatty acid The values for palmitic acid are the mean ± SEM of 8 experiments whereas all the other values represent single experiments

The solutions of fatty acid in serum were subjected to Sephadex column chromatography at room temperature essentially as described by Flodin and Killander (1962) but using a gel bed of 60 ml and 0.9 saline as effluent The void volume was determined with blue dextran with a molecular weight of 500 000 Protein was determined spectrophotometrically at a wave length of 280 mμ The eluted fractions were assayed for radioactivity by liquid scintillation counting

The present investigations were divided into 2 parts One part concerned a comparison between different fatty acids The other part was undertaken to study the effect of an increasing mole ratio fatty acid/albumin

Results

The elution pattern of rat serum proteins (see Fig 1) was similar to that obtained by Flodin and Killander (1962) for human serum proteins Three peaks were found According to the determinations of Flodin and Killander (1962) the first peak represented alfa$_2$ globulin beta$_2$ globulin alfa lipoprotein and beta lipoprotein It coincided with the void volume which equalled 17 ml as was shown in this work by the complete overlapping of the elution curves for dextran and protein (See Fig 1) The second peak of protein consisted of gamma globulin and in the third peak the protein was mainly albumin

The recovery from the Sephadex columns was better than 90 % based on radioactivity measurements Comparison between columns operated at room temperature and at 37 C did not reveal any appreciable difference in the elution pattern of arachidic acid Neither was any difference noted when the 0 2 ml of ethanol used to dissolve the fatty acid salts was evaporated prior to the addition of the serum

Determination of the radioactivity in the eluted fractions (see Table I) showed that labeled fatty acids were eluted with the lipoprotein and the albumin fractions and in the tubes corresponding to 50—70 ml of eluent No peak of radioactivity was found in the gamma globulin fraction

In table I it can be seen that throughout the homologous series of saturated fatty acids there were certain gradual changes in the distribution of radioactivity The caproic acid was found both distributed over the total volume of the column and in the albumin fraction

The capric acid was to more than 90 % eluted with the albumin fraction The acids from lauric to arachidic were to an increasing degree eluted with the lipoprotein fraction

The palmitoleic oleic and linoleic acids were to more than 98 % found in the tubes corresponding to the albumin peak

When the chemical amount of oleic acid was increased a shift in the distribution of radioactivity occurred so that more label was eluted from the column with the lipoprotein fraction

Discussion

The data in the literature dealing with the possible difference in disappearance rate of individual free fatty acids intravenously injected into animals have been somewhat contradictory Originally Fredrickson and Gordon (1958) found no differences in the disappearance rate of palmitic linoleic and oleic acid Later however Uzawa *et al* (1964) compared several fatty acids and noted that oleic acid was more rapidly extracted by the tissues than the other fatty acids Dustin et al (1961) reported that linoleic acid disappeared faster from the blood than palmitic acid Thus it now seems established that the unsaturated fatty acids with 18 carbons are more rapidly extracted than palmitic acid when injected into laboratory animals

This finding was supported by the results of Goransson and Olivecrona (1965) and Goransson (1965 a b c d and e) whose experiments also showed that long saturated fatty acids disappeared more slowly from the blood than shorter saturated fatty acids

In an attempt to add some further information to the discussion of possible differences in the turnover of individual free fatty acids in plasma the present work was undertaken The results suggested that the unsaturated fatty acids in serum were completely albumin bound when used in amounts comparable to those injected earlier into rats The saturated fatty acids with less than 14 carbon atoms were to an increasing degree partitioned in favor of the water phase as might be expected from the decrease in hydrophobic properties of the short fatty acids The long saturated acids were to an increasing extent eluted with the lipoprotein fraction They may therefor have been complexed with the lipoproteins but the fact that the lipoprotein fraction coincided with the void volume makes it impossible from the present data to exclude the possibility of emulsion formation However the agreement of the present data with those of

Laurell (1955) Gordon (1955) Goodman and Shafrir (1959) and Carrol (1964) makes it probable that the fatty acids were in fact complexed with the lipoproteins in the present investigation

In any case the present results indicate differences in the form in which the long saturated and the unsaturated fatty acids exist in the serum studied in this work This discrepancy may not necessarily be primarily determining the rate of disappearance from the blood of the fatty acids in vivo Instead the difference in disappearance and the difference in distribution between the proteins and water phases may both depend on the solubility of the individual fatty acids in water which will be discussed below

The carbon chain of the saturated fatty acids prefers a staggered configuration in hydrophobic milieu With increasing chain length the excluded volume becomes greater This fact together with the concept that the interior of the lipoprotein molecule is less rigid than the interior of the albumin molecule makes a combination with lipoprotein more and more favorable with increasing chain length (Arvidsson 1964)

If it is assumed that the fatty acids have to pass a water phase when transferred from the proteins in the plasma to the surface of the tissue cells and the passage through the water phase is the rate determining step in the kinetic line the rate of uptake of the fatty acids should be dependent on the solubility of each fatty acid in water This hypothesis is supported by the data showing that the long saturated and hydrophobic fatty acids are extracted more slowly than the short and more hydrophilic acids In the same line of observation is the faster extraction of oleic acid than stearic acid and of linoleic acid than oleic acid noted by Göransson and Olivecrona (1965) and Goransson (1965 b and c)

The finding in this work that the short fatty acids were to some extent distributed over the volume of the column is interesting in relation to other results indirectly showing that as much as 50 % of lauric acid and 90 % of capric acid fed to rats may enter the portal circulation whereas fatty acids with a chain length of more than 14 carbon atoms are transported via the thoracic duct incorporated into chylomicrons (Bloom Chaikoff and Reinhardt 1951) Direct evidence of transport of capric acid as such in the portal blood has been presented by Borgstrom (1955) and is also in agreement with the present results

The amount of oleate dissolved in rat serum varied from 0 02 to 10 0 μeq per ml of serum in the present work This corresponds roughly to a mole ratio total fatty acid/albumin of from 2 to 22 assuming normal rat serum to contain 1 μeq of free fatty acid and 30—40 mg of albumin per ml It was found that when the total amount of free fatty acid was approximately 3 moles per mole of albumin virtually all of the added labeled oleic acid was eluted with the albumin When the mole ratio was increased to 6 about 30 of the radioactivity was eluted with the lipoprotein containing globulin peak At a higher mole ratio (approximately 22) an even higher percentage of the radioactivity was eluted with the globulins These findings agree with those of Laurell (1955) and Gordon (1955) who found that an increase of electrophoretic mobility of lipoprotein after addition of increasing amounts of oleate took place at a mole ratio fatty acid albumin equalling 3—4

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Energetics of Isometric and Isotonic Contraction in Isolated Vascular Smooth Muscle under Anaerobic Conditions

By

LENNART LUNDHOLM and ELLA MOHME LUNDHOLM

Received 29 October 1964

Abstract

LUNDHOLM, L. and E. MOHME LUNDHOLM. *Energetics of isometric and isotonic contraction in isolated vascular smooth muscle under anaerobic conditions.* Acta physiol. scand. 1965. 64. 275—282. — In experiments on isolated bovine mesenteric artery the energy production was determined from the lactic acid production under anaerobic conditions. During isometric contraction of the muscle preparations addition of adrenaline or of potassium ions resulted in a 3- to 5-fold elevation of the metabolism concomitant with a rise of tension. When the tension had reached maximal level the metabolic elevation was more moderate — approximately 60 per cent in the experiments with adrenaline and about 20 per cent in those with potassium ions. Thus the muscle consumed more energy in attaining a certain tension level than in maintaining it. Total consumption of high energy phosphate compounds on isometric contraction was estimated to exceed the preformed content by approximately 300 per cent. The energy demand was appreciably greater for isometric than for isotonic contraction both during the increasing tension phase and during maintenance of constant tension. — Dibenamine blocked the metabolic stimulating and the contractile effects of adrenaline but not the effects of potassium ions.

In previous experiments (Lundholm and Mohme Lundholm 1962 a, 1963) we had determined the energy metabolism during isotonic contraction of isolated vascular muscle under anaerobic conditions. It was found that on contraction with catechol amines, histamine, barium ions and electrical stimulation the metabolism increased during shortening of the muscle. The contraction in mm and the increase of metabolism were correlated. Once the muscle had attained a constant degree of contraction the metabolism ceased to increase. Addition of potassium ions was followed by muscle contraction without a coincident stimulation of the lactic acid production. It was nevertheless possible under certain conditions to block selectively the contractile effects of adrenaline and histamine without affecting their stimulatory action on the metabolism (Lundholm and Mohme Lundholm 1963). This somewhat tenuous relation between

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In previous experiments (Lundholm and Mohme Lundholm 1962 a 1963) we had determined the energy metabolism during isotonic contraction of isolated vascular muscle under anaerobic conditions It was found that on contraction with catechol amines histamine barium ions and electrical stimulation the metabolism increased during shortening of the muscle The contraction in mm and the increase of metabolism were correlated Once the muscle had attained a constant degree of contraction the metabolism ceased to increase Addition of potassium ions was followed by muscle contraction without a coincident stimulation of the lactic acid production It was nevertheless possible under certain conditions to block selectively the contractile effects of adrenaline and histamine without affecting their stimulatory action on the metabolism Lundholm and Mohme Lundholm 1963 This somewhat tenuous relation between

TABLE I Isolated bovine mesenteric arteries Tyrode solution with 0.5 per cent glucose in N_2 Effects of adrenaline and K^+ ions on lactic acid production under isometric and isotonic conditions P = probability that the effect was due to chance n = number of tests t/2 = time in min to reach the half maximal value of tension or shortening t = time in min to reach the maximal value Tests with isotonic contraction from Lundholm and Mohme Lundholm (1962) After adrenaline the muscle shortened 1.9 mm (mean) and after K^+ ions 2.0 mm (mean) The muscles were loaded with 10 g

	0—15 min lactic acid production µmol/g/15min			15—120 min lactic acid production µmol/g/105 min				
Drug contraction	control	drug	increase	control	drug	increase	t/2 min	t min
Adrenaline								
isometric	3.7± 0.7	10.5± 0.5	6.8± 1.0	32.7± 1.1	52.7± 2.7	20.0± 3.2	1.8± 0.2	6.4± 0.6
n=6			P<0.001			P<0.01		
isotonic	5.1± 1.4	6.8± 1.3	1.7± 0.6	28.9± 3.2	28.9± 3.3	0.3± 2.7	1.7± 0.1	9.2± 0.4
n=7			P<0.05					
K^+ ions								
isometric	2.9± 0.5	12.1± 1.3	9.2± 1.2	29.8± 1.9	35.3± 1.7	5.5± 1.4	1.3± 0.14	7.8± 0.9
n=5			P<0.01			P<0.02		
isotonic	5.1± 1.4	2.1± 1.1	-2.8± 0.5	28.6± 3.0	27.4± 1.2	-1.2± 3.2	0.6± 0.03	6.3± 0.6
n=7			P<0.001					

metabolic stimulation and isotonic contraction prompted us to study the metabolic behaviour during isometric contraction We elected for this purpose to investigate the effects of adrenaline — on which much of our previous research had focussed — and of potassium ions which under isotonic conditions did not stimulate the metabolism.

Methods

For these experiments we used bovine mesenteric artery prepared and treated as described in an earlier paper (Lundholm and Mohme Lundholm 1962 a) The preparations were mounted in specially designed frames in which the length could be measured and adjusted by means of a micrometer screw thus assuring length uniformity of all preparations at the start of the experiment. A description of the frame will be published (Lundholm and Mohme Lundholm 1965 b) The preparations were 14 mm wide 10—12 mm long and approximately 1 mm thick, the weight being about 0.15 g Tension was recorded by isometric pen on smoked paper Re

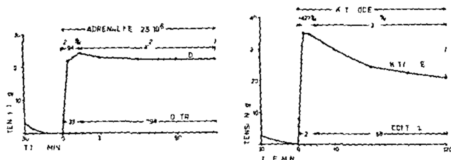


Fig 1 Effects of adrenaline and K Tyrode on tension and lactic acid production of isolated bovine mesenteric artery in Tyrode solution containing 0.5 per cent glucose and bubbled with nitrogen Lactic acid production is indicated both in percent of the control values and in mg/100 g muscle during the periods 0—15 min and 15—120 min after addition of the drug

cording was not absolutely isometric at a developed tension of 25 g the muscle shortened 0.5 mm Prior to the addition of adrenaline or of potassium ions the tension was adjusted to an initial value of 2 g

The lactic acid content of muscle preparation and suspension solution was assayed by a previously reported enzymatic method involving the use of lactic acid dehydrogenase (Lundholm Mohme-Lundholm and Vamos 1963 Lundholm Mohme-Lundholm and Svedmyr 1963)

Other methodologic data were as follows For determining the time pattern of lactic acid production on isometric contraction after adrenaline and potassium ions eight preparations were immersed in organ baths containing Tyrode solution bubbled with nitrogen At the end of 30 min two preparations were taken for determination of the initial lactic acid content and the solution from the other six was exchanged for a similar solution containing 0.5 per cent glucose To two of these six was added adrenaline at a concentration of 2.5 10 and to two others a Tyrode solution containing instead of sodium salts equimolar amounts of potassium salts (In the following the respective solutions will be designed as Na Tyrode and K Tyrode) The remaining two preparations served as controls For lactic acid assay of the muscle and its suspension solution one control one adrenaline and one K Tyrode preparation were taken after 15 min and the remaining one of each after 120 min The difference between the initial level and the summed lactic acid content of preparation and solution on termination of the experiment constituted the lactic acid production

In the dibenamine experiments 4 of 8 preparations were first treated for 60 min with dibenamine at a concentration of 5 10 in Tyrode solution containing 0.5 per cent glucose and bubbled with nitrogen One untreated and one dibenamine treated preparation were then taken for determination of the initial lactic acid content whereupon the suspension solution was changed To one each of the three dibenamine treated preparations was added adrenaline at a concentration of 2.5 10 or K Tyrode or NA Tyrode containing 0.5 per cent glucose

Results

Effect of Adrenaline and K Tyrode on Lactic Acid Production and Tension The results of the experiments with adrenaline are summarized in Table I and Fig 1 During the initial 15 min period of developing tension the lactic acid production rose by approximately 300 per cent During the next 105 min the tension remained near its maximal level and the metabolism though significantly lower than the 15-min value was still elevated by about 60 per cent

TABLE II The effect of dibenamine on the stimulating action of adrenaline or K ions on lactic acid production and tension of isolated bovine mesenteric arteries Tests in N_2 and 0.5 glucose under isometric conditions Mean of 5 tests

Drug concentration g/ml	Lactic acid production µmoles/g/120 min Control value	Change after drug	Maximal tension g
Adrenaline 2.5 10	31.7±2.2	+22.0±4.5 P< 0.01	27
K Tyrode	31.7±2.[illegible]	+10.6±2.9 P< 0.05	29
Dibenamine 1 10 *	31.7±2.2	+2.0±5.0	0
Dibenamine 1 10 + Adrenaline 2.5 10 *	33.7±1.0	−0.3±0.8	0
Dibenamine 1 10 + K Tyrode	33.7±1.0	+7.4±2.0 P< 0.0[illegible]	25

After K Tyrode too the lactic acid production rose substantially — by almost 500 per cent — during the first 15 min (Table I and Fig 1) For this period the maximal tension and the stimulation of lactic acid production showed a certain proportionality The quotient $\frac{\text{maximal tension in g}}{\text{lactic acid increase in mg/100 g/ 15 min}}$ was 0.44 in the adrenaline experiments and 0.43 in the experiments with K Tyrode In the latter the tension after reaching maximal level gradually declined At 120 min the lactic acid production was elevated by only 20 per cent — a value significantly lower than that for the first 15 min

Influence of Dibenamine upon the Contractile and Metabolic Stimulating Effects of Adrenaline and K Tyrode Since bovine mesenteric arteries contain relatively substantial amounts of noradrenaline (Schmiterlow 1948) it was conceivable that the potassium ions had elicited some of their effects by liberating noradrenaline from the vascular tissue It was found however that dibenamine at a concentration of 5 10 selectively inhibited the contractile and metabolic stimulating effects of adrenaline without influencing the corresponding effects of potassium ions (Table II) hence it seemed likely that adrenaline and potassium ions had produced their effects via an action upon different receptors

Degree of activation in isometric and isotonic contraction In arterial muscle an isometric contraction was associated with far greater stimulation of the metabolism than was an isotonic contraction (Table I) This disparity is discussed more fully in a later section of this paper A possible explanation however was that either a smaller number of muslce fibres were activated or the degree of activation was less with isotonic than with isometric contraction To test this hypothesis two preparations of equal length from the same artery were contracted with adrenaline — one of them isotonically and the other

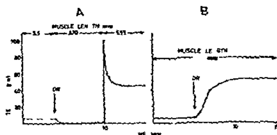


Fig 2 Mesenteric artery in Tyrode solution bubbled with N_2 0.5 glucose Tension measured with force transducer on Grass polygraph A The tension was 6 g at an initial length of 5 55 mm The muscle was then allowed to shorten to 3 70 mm at which length the muscle gave a just detectable tension after addition of adrenaline (10) The muscle was then rapidly (< 2 sec) elongated to 5 55 mm and the change of tension measured B The same artery at a constant length of 5 55 mm tension development after addition of adrenaline

isometrically (Fig 2) When the isotonic preparation had shortened to a constant value it was rapidly elongated to its original length i e the same length as the isometric preparation The tension of the formerly isotonic preparation increased thereby to a value two to three times that of the originally isometric preparation and then gradually declined to the same level This experiment indicated that the number of activated muscle fibres and/or the degree of activation were the same for the two preparations

Discussion

Lactic Acid Production as a Criterion of Energy Metabolism The extent to which the lactic acid production under anaerobic conditions reflects the total energy metabolism during contraction is an important question Another series of experiments was therefore conducted (Lundholm and Mohme Lundholm 1965 a) in order to elucidate the effect of adrenaline on the high energy phosphate compound content (adenosine triphosphate and creatine phosphate) of arterial muscle during isometric contraction Seven minutes after addition of adrenaline the relevant content had fallen by an average of 0 7 μeq/g and at the end of 60 min it was still depressed by 0 5 μeq g — For each mole of lactic acid produced from glucose 1 eq of high energy phosphate compound is synthesized concomitantly — On isometric contraction in the present experiments the lactic acid production increased during the first 15 min by 6 8 μmoles g corresponding to synthesis of 6 8 μeq g high energy phosphate compounds Since as mentioned above the content fell simultaneously by 0 7 μeq g the total consumption of high energy phosphate compounds was 7 5 μeq g During the period from 15 to 120 min the lactic acid production reflected the energy metabolism fairly accurately inasmuch as the high energy phosphate compound content probably remained constant

Energy Metabolism on Isometric Contraction The arterial muscle can therefore be estimated to have consumed during isometric contraction by adrenaline approximately 7 5 μeq g high energy phosphate compounds over and above the basal metabolism. This consumption was about three times the preformed amount the latter being in the region of 2 5 μeq g Lundholm and Mohme Lundholm 1962 b) A decrease in the

adenosine triphosphate and creatine phosphate contents has as pointed out above been demonstrated on isometric contraction The preformed high energy phosphate compound content is apparently insufficient for maximal isometric contraction of vascular muscle such contraction depends rather on a continuous energy production

Time Pattern of Energy Metabolism The lactic acid production was appreciably higher during the initial phase of contraction, when tension was being produced than subsequently when a constant tension was maintained The difference was particularly marked on contraction with K ions Vascular muscle thus required a greater amount of energy for the production than for the maintenance of tension Similar findings have been reported for frog striated muscle (Hartree and Hill 1920 Hill and Woledge 1962) and for snail smooth muscle (Bozler 1930) In this respect the behaviour of various types of muscle appears to be qualitatively similar A quantitative comparison of frog muscle and vascular muscle with regard to the energy required for producing a given degree or tension will be reported in a subsequent paper (Lundholm and Mohme Lundholm 1965 a) in which the energy consumption is determined by a method that permits comparison with Hill s thermo-electric measurements It can be mentioned here however that the tension production energy consumption appears to be of similar magnitude for different types of muscle whereas the tension maintenance energy consumption is appreciably lower for vascular smooth muscle than for frog striped muscle

There could be an alternative explanation of the fact that the energy demand of vascular muscle was lower for maintenance than for production of tension. The initial elevation of tension after adrenaline and potassium ions probably reflected the degree to which the muscle was in an active state On the other hand the maintenance of constant tension following the initial phase was not necessarily dependent on this active state but could have been due to a more passive tonic mechanism The experiments with K ions in which the tension fell after having reached maximal level suggested that the active state may change with time Further experiments were therefore conducted (Lundholm and Mohme Lundholm 1965 b) to determine the degree of active state 10—120 min after addition of adrenaline or of potassium ions

With adrenaline under anaerobic conditions the active state remained constant throughout the experiment but with potassium ions it decreased somewhat Assuming that stimulation of the metabolism was elicited and was commensurate with the degree of active state the lower energy consumption for the 15—120 min period after adrenaline could hardly have been attributable to depression of the active state In the potassium ion experiments on the other hand the reduced energy metabolism was probably ascribable in some measure to a depressed active state Over the period from 15 to 120 min the energy metabolism was less elevated in the potassium ion than in the adrenaline experiments The main reason the energy metabolism was lower once the tension attained a fairly constant level was doubtless that the muscle consumed less energy in maintaining than in producing tension

Energy Metabolism in Isotonic and Isometric Contraction of Vascular Muscle On thermo-electric determination of the energy metabolism of frog striated muscle Hill (1938) observed that isotonic contraction developed more energy than did isometric During isotonic contraction the heat of shortening was added to the heat of activation No definite metabolic counterpart of heat of shortening has however been demonstrated on determination of the energy metabolism from the oxygen consumtion (Whalen 1962) or from the hydrolysis of creatine phosphate (Mommaerts Seraydarian and Marechal 1962 Cain Infante and Davies 1962 Carlson Hardy and Wilkie 1963)

Hill (1964) has shown moreover that the heat of shortening was probably over estimated in earlier studies

In arterial muscle nevertheless isometric contraction — as is clear from table I — was associated with far greater stimulation of the metabolism than was isotonic contraction During the first 15 min which coincided rougthly with the initial contraction phase the metabolic increase following adrenaline was several times greater in the isometric than in the isotonic experiments Indeed in the former experiments but not in the latter the metabolism remained elevated even after the tension had reached a fairly constant level

On contraction of the muscle with potassium ions the lactic acid production showed no increase at all under isotonic conditions We had earlier observed that adrenaline stimulated the anaerobic lactic acid production of vascular muscle after its contractile effect had been blocked by dihydroergotamine (Lundholm and Mohme Lundholm 1963) This lactic acid production was equal in magnitude to that associated with isotonic contraction but was substantially lower than that associated with isometric contraction The high energy phosphate compound content declined when isotonic contraction was elicited by adrenaline but rose when the latter s contractile effect had been blocked by dihydroergotamine (Beviz and Mohme Lundholm 1965) — Under isotonic conditions therefore contraction is doubtless an energy requiring process indeed a linear relation between lactic acid production and contraction has been demonstrated It is nevertheless probable that the energy consumption of the contractile process accounted for only a part of the lactic acid production shown for isotonic contraction in Table I Where a correction made accordingly however it would add to the disparity in terms of energy metabolism between isotonic and isometric contractions

To what may this disparity be attributed Since the time pattern was almost identical for the two types of contraction (see Table I) differences in duration of the contractions could scarcely have been implicated Furthermore the respective initial lengths of the muscle preparations were practically equal The degree of activation and/or the number of activated muscle fibres were probably the same for the two types of contraction (Fig 2)

One difference between frog striated muscle and vascular muscle was however that whereas the former contracted isotonically from its fully relaxed state the arterial muscle was already contracted to some extent at the outset For example the preparations in Table I contracted isotonically only 2 mm whereas maximal contraction from the fully relaxed state may theoretically at least amount to some 9 mm (Lundholm and Mohme Lundholm 1962 a) In these earlier experiments we also demonstrated a linear relation between the metabolic increase and the contraction in mm If assuming this relation to be a factual one and if the muscle had contracted 9 mm instead of 2 mm the lactic acid production would have been four and a half times as great i e approximately 7 7 μmoles/g — a value somewhat exceeding that for isometric contraction Before any final stand is taken regarding the question whether vascular muscle requires more energy for isometric than for isotonic contraction it would be advisable therefore to determine the energy metabolism for each type of contraction from the fully relaxed state

We are grateful to the assistance of Mr Sandor Vámós Financial support has been given by the Swedish State Medical Research Council and the Swedish National Association against Heart and Chest Diseases

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Uptake of Monoamines by Mesenteric Mast Cells of the Mouse

By

OLAVI ERANKO and LAURI KAUKO

Mammalian mast cells have been demonstrated to contain histamine (H) 5 hydroxy tryptamine (5 HT) and/or dopamine (DA) (Lagunoff *et al* 1961 Adams Ray *et al* 1964) Using radioactive isotopes Furano and Green (1964) showed that rat s mast cells take up exogenous 5 HT and H but not DA or noradrenaline (NA) Adams Ray (1964) presented histochemical evidence that mast cells of many mammals take up large amounts of dihydroxyphenylalanine (D) but not DA although D apparently is after uptake converted into DA in the mast cells Observations to be presented in the present paper indicate that mast cells of the mesenterium of the mouse are capable of concentrating not only 5-HT and D but also DA and NA

Albino mice weighing about 30 g were intraperitoneally injected with 1 5—3 mg of amine or D in 0 3 ml of saline After 5—16 hours the mice were killed and the mesenterium was stretched on a slide dried and exposed to formaldehyde vapour for 1—15 hours at 80 C to convert the amines into fluorescent compounds (Falck 1962 Eranko 1964) After fluorescence photomicrography the preparations were stained with toluidine blue and photographed again now in transmitted visible light

Fig 1 shows the mesenterium of a normal mouse injected with saline only No or very little monoamine fluorescence is visible although several mast cells are present as can be seen from Fig 2 Fig 3 is from the mesenterium of a mouse given 1 5 mg of NA 12 hrs before killing Intensely fluorescent cells are present and Fig 4 proves these to be mast cells

After injection of D DA or NA a green fluorescence appeared in the mast cells while the fluorescence observed after 5-HT administration was yellow green Exposure for 1 hour to formaldehyde was enough to develop the fluorescence Injections of adrenaline (A) or H failed to make the mast cells fluorescent even if the specimens were exposed overnight to formaldehyde because A is less readily converted into a fluorescent compound than other amines (Falck 1962 Eranko 1964)

The observations indicate that the mast cells in the mesenterium of the mouse contain very little if any intrinsic D DA NA 5 HT or A although they are capable of taking up and concentrating all of these compounds but A It has been claimed that H is not demonstrable with the method employed (Falck 1962) Therefore it cannot be concluded with certainty whether the mast cells examined contain and/or take up H

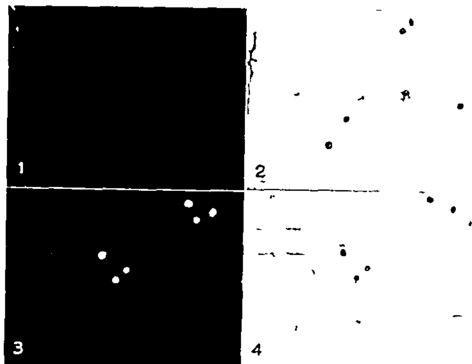


Fig 1 Formaldehyde induced fluorescence in the mesenterium of a control mouse The whole field is essentially non fluorescent 200 ×

Fig 2 The same field as that in Fig 1 after staining with toluidine blue Several mast cells are intensely stained 200 ×

Fig 3 Formaldehyde induced fluorescence in the mesenterium of a mouse injected intra peritoneally with 1 5 mg of noradrenaline 15 hours before killing Several intensely fluorescent cells are visible 200 ×

Fig 4 The same field as that in Fig 3 after staining with toluidine blue The fluorescent cells can be identified as intensely basophil c mast cells 200 ×

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Adrenergic Synaptic Terminals in Autonomic Ganglia

By

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Adrenergic synaptic terminals as demonstrated by the histochemical fluorescence method of Falck and Hillarp have been reported in both sympathetic (Hamberger Norberg and Sjoqvist 1963) and parasympathetic ganglia (Norberg 1964 Hamberger and Norberg 1965) The present results indicate that adrenergic synapses constitute important and widespread systems in the autonomic ganglia

Autonomic ganglionic tissues from large numbers of untreated cats rabbits and rats (Sprague Dawley) were collected during an investigation with the above mentioned histochemical method on the sympathetic adrenergic neuron (for details on the procedures used see Norberg and Hamberger 1964) In 3 rabbits preganglionic denervation was performed on the superior cervical ganglion 3 to 5 days before dissection

The *superior cervical ganglia* in the rabbit contain numerous adrenergic varicose terminals which form prominent basketlike synaptic structures around ganglion cells (Fig 1) and persist after preganglionic denervation In the rat the synaptic connections in these ganglia are made mainly with cell processes presumably dendrites No definite proof has been obtained for the existence of adrenergic synapses in this ganglion in the cat In the *stellate ganglia* of the rat adrenergic synaptic structures have been found to be of the same type as in the superior cervical ganglia making contacts mainly with cell processes The *celiac and inferior mesenteric ganglia* have the most well-developed systems of terminals of all the sympathetic ganglia Very prominent basketlike synaptic structures have been found in the rabbit as earlier reported for the cat (Hamberger Norberg and Sjoqvist 1963) The *intramural ganglia of the intestines* have been found to contain adrenergic synaptic structures in the rabbit — and also in guinea pig and man (Norberg unpubl obs) — as previously shown for the rat and the cat (Norberg 1964) The *ciliary ganglion* of the cat contains distinct adrenergic synaptic structures (Fig 2) but these seem to be rather few Earlier pharmacological data indicate the presence of adrenergic synapses in ciliary ganglia of the dog (Tum Suden *et al* 1951)

Although adrenergic synaptic terminals have not at present been found with certainty in all types of autonomic ganglia and their occurrence may differ as between species it cannot be ruled out that they are in fact considerably more widespread than demonstrated Certain technical difficulties seem to be involved as the terminals in *e g* the superior cervical ganglion of the rat are often very difficult to visualize The slightest diffusion of the adrenergic transmitter probably makes the very fine terminals completely undetectable particularly if they are in contact with fluorescent cell bodies or processes

The observations reported here do not in themselves permit any definite conclusions as to the function of these systems of adrenergic synapses in the autonomic ganglia In



Fig 1

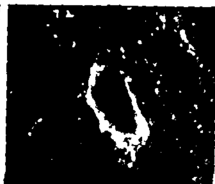


Fig 2

Fig 1 Sup cerv ganglion rabbit preganglionic denervation 5 days before dissection × 330
Fig 2 Ciliary ganglion cat × 330
One of the gangli n cells in each Figure is surrounded by a basketl ke synaptic structure made up of numerous adrenergic varicose terminals lying together This arrangement makes the individual varicosities difficult to distinguish In Fig 1 the cellular fluorescence is of somewhat lower intensity than usually

conjunction however with the evidence (see Marrazzi 1939 Lundberg 1952 McDougal and West 1954 McLennan 1963) indicating that adrenaline and noradrenaline may produce an inhibition of the ganglionic transmission and that acetylcholine is an excitatory transmitter in the sympathetic ganglia the results strongly suggest that their functions are inhibitory

This investigation has been supported by research grants (Y 247 and 482) from th Swedish Medical Research Council by a L S Public Health Service Research Grant (NB 03736-01) from the National Institute of Neurological Diseases and Blindness and by a research grant from Magn Bergvalls Stiftelse Stockholm Sweden

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Acta physiol scand 1965 64 287—288

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Catecholamines in the Preaortal Paraganglia of Fetal Rabbits

By

Tomas Brundin

The morphological development of the adrenal medulla and the paraganglia in various mammals was described by Kohn (1903) In rabbits he found that the upper parts of the largest preaortal paraganglia extend to and become enclosed by the adrenal cortex Thus at birth the adrenal medullae of both sides are connected with one another via the paraganglionic tissue Concerning the hormone content of these organs it has been held that noradrenaline is the only catecholamine formed during fetal life (West 1955) Recent investigations have shown however that the main catecholamine in the adrenal medulla of rabbit fetuses during the last third of their intrauterine life is adrenaline (Brundin 1965) In the study to be reported here it was found that the hormone distribution in the preaortal part of this organ complex in rabbits distinctly differs from that of the adrenomedullary part

Pregnant albino rabbits were killed by a blow on the head 29 days after conception The fetuses were immediately taken out and decapitated The large preaortal para ganglia and the adrenals were removed and separated under a dissection microscope The catecholamine contents of the tissues were determined fluorimetrically

The analysis showed (Fig 1) that in the adrenals about 70 per cent of the catechola mines was adrenaline while the paraganglionic part connecting the adrenals contained almost exclusively noradrenaline Thus different parts of what appears to be the same morphological structure showed remarkable differences in hormonal contents suggest ing different functions of the adrenal medulla and the paraganglia

A preganglionic innervation of the paraganglia resembling that of the adrenal medulla has been morphologically described by Hollinshead (1937) Work is in progress on the physiological significance of this innervation during the prenatal and newborn period

This work has been supported by grants from Stiftelsen Lars Hiertas Minne and from Svenska Sallskapet for Medicinsk Forskning

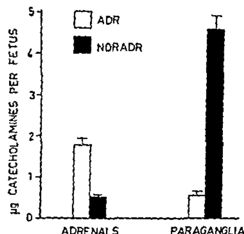


Fig 1 Mean values of adrenaline and noradrenaline in adrenals and paraganglia from 6 fetal rabbits at 29 days gestation Vertical bars standard error of mean

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Studies on the Peripheral Circulation and Metabolism in Man

IV. Oxygen Utilization and Lactate Formation in the Legs of Healthy Young Men during Strenuous Exercise

By

BENGT PERNOW, JOHN WAHREN and STAFFAN ZETTERQUIST

Received 16 October 1964

Abstract

Pernow, B., J. Wahren and S. Zetterquist. *Studies on the peripheral circulation and metabolism in man. IV. Oxygen utilization and lactate formation in the legs of healthy young men during strenuous exercise.* Acta physiol. scand. 1965. 64. 289—298. — 19 healthy young men exercised on a bicycle ergometer with increasing work loads. From indwelling catheters in the brachial artery and the femoral vein blood samples were drawn before, during and after exercise. The a—v O_2 difference in the leg increased linearly in relation to heart rate and O_2 uptake to a mean value of 163 ml/l at maximal work. At this intensity (heart rate 180—19[illegible] beats per min) the mean value of the venous O_2 saturation was 9 per cent. A linear relationship existed between log lactate and heart rate during work as well as between log lactate and O_2 saturation of the femoral venous blood. The slope for the semilogarithmic regression of the femoral venous concentration of excess lactate (XL) on heart rate was significantly steeper than that of venous total lactate concentration. pH of venous blood decreased during exercise to a mean value of 7.09 at maximal load. A linear relation was obtained between decrease in pH and increase in lactate. The reproducibility of the abovementioned results was evaluated for 6 subjects. A comparison between exercise performed with one and two legs showed that heart rate increased almost identically in relation to O_2 uptake at both types of exercise. The venous O_2 saturation was somewhat lower at submaximal work loads during one leg exercise, while the final venous O_2 saturation values at maximal work were almost identical. Lactate increased more rapidly during one leg exercise. These data seem to indicate that blood flow in relation to O_2 demand is less adequate in one leg than in two-leg exercise at comparable working intensities.

During exercise performed on a bicycle ergometer with successively increasing working intensity the femoral venous oxygen saturation decreases linearly (Carlson and Pernow 1961). At a moderate work a mean value of about 20 per cent was observed (Donald *et al.* 1957, Carlson and Pernow 1961 and Reeves *et al.* 1961). However, a more complete extraction of oxygen is obtained when the blood flow response to exercise is impaired by disease. Thus Donald *et al.* (1957) showed that in patients with mitral disease the femoral oxygen saturation often decreased to values below 5 per cent even during light exercise. Similar findings have been made in patients with arterial occlusive disease (Carlson and Pernow 1962, Bellman, Pernow and Zetterquist 1962).

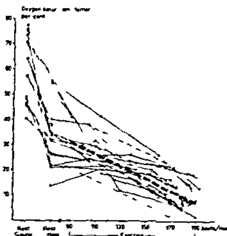


Fig 1

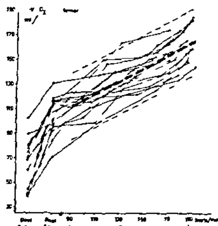


Fig 2

Fig 1 Oxygen saturation in femoral venous blood of subjects at rest supine sitting on the bicycle and performing two-leg exercise correlated to heart rate The heavy dotted line indicates the regression line ($y = 52.7 - 0.24x$, $r = 0.80$xxx) for the values during work with ± 2 SD within the thinner dotted lines

Fig 2 Arterial venous oxygen difference in the leg of subjects at rest, supine sitting at the bicycle and performing two-leg exercise correlated to heart rate The heavy dotted line indicates the regression line ($y = 67.8 + 0.50x$, $r = 0.69$xxx) for the values during work with ± 2 SD within the thinner dotted lines

The aim of the present investigation was to study whether an almost complete utilization of oxygen in the legs is obtained also in normal subjects in connection to a maximal work. The influence of the mass of muscles taking part in the work was studied by performing exercise tests on a bicycle with one and both legs respectively along the same line as reported by Dunér (1959) The changes in blood lactate pyruvate and pH were measured in order to compare the aerobic and anaerobic components during exercise of varying degrees. Finally the reproducibility of the metabolic results was studied by repeated exercise tests

Material

19 healthy well trained firemen aged 22—42 years participated Each had recently undergone a clinical examination and been found normal.

Procedure and methods

The investigations were performed in the morning The fasting subjects exercised on a bicycl ergometer The experimental conditions th procedure during the course of the investigation and the methods of analysis were all identical with those previously reported (Carlson and Pernow 1961 1962

When the teflon catheters had been inserted percutaneously into a femoral vein and a brachial artery the subjects rested for half an hour Blood samples were then collected for resting values 5 min after the subject had taken his place on the bicycle a new blood sample was drawn from the femoral vein with th sampled leg hanging straight down The exercise began at 300 kpm min and increased every 5th min by a further 300 kpm min up to a maximal load of 1,200—1,800 kpm min The pedal speed was 60 r p m. At the end of each load blood samples were drawn simultaneously from both catheters Oxygen uptake in the lungs was followed during exercise in 11 subjects. The heart rate was calculated from electrocardiographic records.

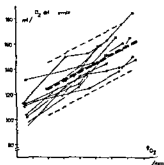


Fig 3 Arterio-venous oxygen difference in the leg of subjects at rest supine performing a two-leg exercise correlated to oxygen uptake in the lungs The dotted lines indicate the regression line ($y = 110.5 + 13.6x$ $r = 0.69$) ± 2 SD

Results

Oxygen (Fig 1—3 Table I) A significant decrease in O_2 saturation of the femoral venous blood was obtained at rest by changing the position from supine to sitting ($p < 0.001$) The further decrease in femoral venous O_2 saturation during exercise as well as the increase in a—v O_2 difference was linear in relation to both heart rate (Fig 1—2) and O_2 uptake in the lungs (Fig 3) At the highest work load when the heart rate was 180—195 beats per min the O_2 saturation in blood from the femoral vein was 2—16 per cent (mean value 9) and the a—v O_2 difference 145—188 ml/l (mean value 163) After work the O_2 content and satuation of the femoral venous blood increased rapidly In 5 cases blood was drawn within 30 sec after the end of work with the subjects still sitting on the bicycle The O_2 saturation of these samples had increased from 5 to 11 per cent (mean value 8) at the end of work to 20—35 per cent (mean value 27) It then further increased to values probably significantly higher than at rest before work Thus the p-value for the difference in O_2 saturation before work and 3 to 5 min after work was < 0.02

Lactate (Fig 4 Table I) The increase in log for arterial and venous lactate concentrations during work was linear in relation to heart rate The regression coefficients for the two relations did not differ significantly ($p > 0.1$) At maximal work the lactate concentration of the arterial blood was 7 7—12 5 mmoles/l (mean value 10 3) and of the femoral venous blood 8 5—14 6 mmoles/l (mean value 10 8)

A significant correlation was obtained between the anaerobic and aerobic components of the total muscular metabolism This relationship is indicated in Fig 6 where the logarithm for femoral venous lactate is plotted against the O_2 saturation of the same blood samples

The arterial lactate concentration remained unchanged during the first minutes after work compared with the levels at the end of work (10 3 mmoles/l at the end of work compared with 9 6 mmoles/l 3 min later mean values $p > 0.4$) The venous lactate however increased slightly after the exercise Thus the venous lactate at maximal work was 10 8 mmoles/l and 11 6 3 min after work This difference was probably significant ($p < 0.05$) The venous arterial lactate difference was highly significant both at maximal work and 3—5 min after the end of work ($p < 0.001$)

Pyruvate (Table I) Pyruvate concentration increased gradually during exercise and reached at the end of work 0 19—0 34 mmoles/l (mean value 0 28) in arterial blood and 0 24—0 36 mmoles/l (mean value 0 33) in venous blood The difference in arterial and venous pyruvate at maximal work was highly significant ($p < 0.001$) The pyruvate

TABLE I Mean value (M) and standard deviation (SD) for variables measured at a two l g

		At rest supine	At rest sitting
Femoral venous oxygen saturation per cent	M	60	30
	SD	12	11
	N	19	19
Arterial venous oxygen difference ml/l	M	65	98
	SD	20	14
	N	19	19
Venous pH	M	7 40	
	SD	0 02	
	N	12	
Arterial lactate mmoles/l	M	0 8	
	SD	0 2	
	N	16	
Femoral venous lactate mmoles/l	M	0 9	
	SD	0 2	
	N	19	
Arterial pyruvate mmoles/l	M	0 03	
	SD	0 03	
	N	17	
Femoral venous pyruvate mmoles/l	M	0 10	
	SD	0 02	
	N	16	

concentration continued to increase immediately after work and was 3 min after work 0 26—0 59 mmoles/l in the venous blood (mean value 0 43) This increase in pyruvate during the first 3 min after work was significant ($p < 0 01$)

Excess of lactate (XL) XL was calculated for arterial and venous blood according to the equation given by Huckabee (1958) $XL = (L_n - L\ ) - (I_n - I\ )\ (L\ /P\ )$ where L_o and P_o are the lactate and pyruvate concentrations at rest and L_n and P the corresponding values during exercise

The XL values for femoral venous blood increased during exercise in a manner similar to lactate i e a very small increase at slight and moderate work and a steep rise as soon as the work became heavy As for log lactate concentration the increase of log venous XL was linearly correlated to the heart rate during work (Fig 4 and 5) The slope of the semilogarithmic regression for venous XL on heart rate was however significantly steeper than the corresponding value for venous lactate on heart rate ($p < 0 001$) and probably significantly steeper than the slope of the regression for log arterial XL on heart rate ($p < 0 05$) The regression line of log arterial XL on heart rate however paralleled that of arterial lactate on heart rate The latter regression equations were $y = 0 013x - 1 61$ and $y = 0 011x - 1 05$ respectively

exercise in normal subjects N = number of observations

During exercise heart rate				After exercise min		
100	130	160	180–195	3	5	10
26	29	35	9	75	71	69
10	6	5	2	14	13	11
19	19	19	13	10	10	10
116	131	147	163			
12	14	12	10			
18	18	18	11			
	7 31	7 25	7 09			
	0 04	0 05	0 05			
	12	17	10			
	2 5	5 2	10 3	9 6	8 6	8 3
	0 8	2 0	1 9	2 5	2 9	1 9
	9	14	10	10	8	8
	2 9	5 5	10 8	11 6	9 1	7 7
	1 0	1 5	1 9	2 3	2 1	2 1
	13	18	10	10	10	10
	0 15	0 20	0 28	0 37	0 39	0 43
	0 04	0 04	0 05	0 03	0 08	0 04
	13	15	10	10	8	8
	0 19	0 24	0 33	0 43	0 41	0 40
	0 05	0 06	0 05	0 11	0 10	0 03
	17	16	10	10	6	10

pH The pH of femoral venous blood was 7 35—7 42 at rest (mean value 7 40) During work the blood became increasingly acidified the lowest pH values (6 93—7 26 mean value 7 09) being recorded at maximal work and immediately after the end of work A linear correlation was obtained between pH and lactate concentration of the femoral venous blood during work (Fig 7)

Comparison between one leg and two-leg exercise

Four subjects exercised twice with an interval of 1 1/2 hr each time up to maximal levels Subjects R P M B and P L worked first with one leg and then with both legs Subject K H started with the two-leg work The results are given in Fig 8 in which the work intensities are shown in terms of oxygen uptake The increase of heart rate in relation to O_2 uptake was in 3 cases almost identical in the two types of work while a higher heart rate was recorded in the one leg exercise in case K H The femoral venous O_2 saturation was always somewhat lower when moderate work was performed with one leg than with both At maximal work however the final O_2 saturation values were almost identical in the two types of work A corresponding pattern appeared for the a—v O_2 difference

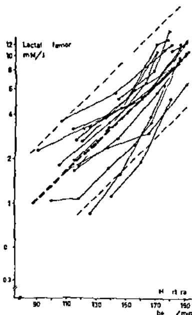


Fig 4

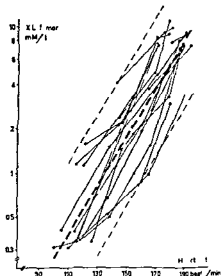


Fig 5

Fig 4 Log lactate concentration of femoral venous blood during exercise in relation to heart rate The heavy dotted line represents the regression obtained from the equation $y = 0\ 010x - 0\ 86$ $r = 0\ 83^{xxx}$ The thinner dotted lines indicate $\pm$ 2 SD

Fig 5 Log excess of lactate (XL) of femoral venous blood during exercise in relation to heart rate The heavy dotted line represents the regression obtained from the equation $y = 0\ 017x - 2\ 27$ $r = 0\ 88^{xxx}$ The thinner dotted lines indicate $\pm$ 2 SD

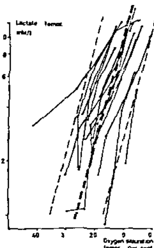


Fig 6

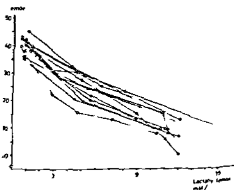


Fig 7

Fig 6 Lactate concentration of femoral venous blood during exercise in relation to the oxygen saturation of the same blood samples The heavy dotted line represents the regression obtained from the equation, $y = 1\ 65 - 0\ 061x$, $r = 0\ 52$ xx The thinner dotted lines indicate $\pm$ 2 SD

Fig 7 pH of femoral venous blood during exercise correlated to lactate concentration of femoral venous blood

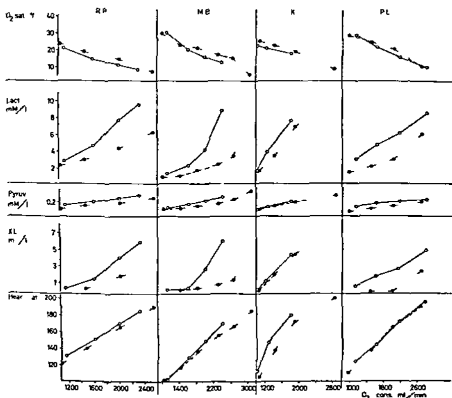


Fig 8 Changes in femoral venous oxygen saturation lactate pyruvate and XL-concentrations and of heart rate during exercise performed with one leg (○———○) and two legs (● ●)

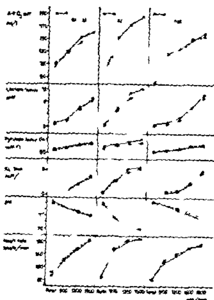
A definite difference was observed in the increase of lactate during the two types of work The lactate concentration of the femoral venous blood was thus always higher in the one leg exercise than in the two-leg exercise at comparable working intensities This finding is in agreement with that of Dunér (1959) for arterial blood A similar pattern was found for pyruvate and XL

Reproducibility of the metabolic results

In order to evaluate the normal biological variation in a–v O_2 difference and oxygen saturation lactate and pyruvate concentration and pH of the femoral venous blood at rest and during exercise two identical exercise tests were performed by the same subjects at an interval of either one year or 1 / hr

3 subjects (Fig 9) exercised on a bicycle in exactly the same manner with one year s interval The blood volume and physical working capacity were the same before both tests As is seen from the figure the differences in the measured parameters were strikingly small

Three other subjects (Fig 10) performed two identical exercise tests at a 1 / hr interval Between the work periods the subjects rested in the supine position and drank water



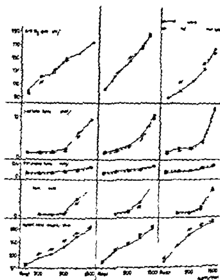


Fig 9 Fig 10

Fig 9 The arterio-venous oxygen difference in the leg the femoral venous concentrations of lactate, pyruvate and XL, the femoral venous pH and the heart rate at rest and during two identical tests (one year interval) in three healthy males

Fig 10 The arterio-venous oxygen difference in the leg the femoral venous concentrations of lactate pyruvate and XL, the femoral venous pH and the heart rate at rest and during a two-leg exercise in three healthy males with the work identically repeated after 90 min

ad lib Exactly the same work could be performed on both occasions However all subjects found the highest load in the second test to be much heavier and more exhausting than in the first The heart rate during exercise was generally higher in the second test. This was more obvious during submaximal work, two subjects showing a difference in heart rate of 15 to 20 beats min The difference became somewhat smaller as exercise continued The mean difference in femoral venous oxygen saturation was 0 5 per cent at the highest loads the corresponding value for a v O_2 difference was 12 ml l In two cases (R. R and B I in Fig 9) the total lactate concentration and XL of femoral venous blood were about 1 mmoles/l lower in the second test at identical work load whereas no such difference was found in the third case

Discussion

In this series of studies it has been evaluated to what extent the availability of O_2 during muscular exercise of different intensities is adequate in relation to the oxygen demand of the working tissue This has been based on measurements of the O_2 content and concentration of lactate and pyruvate in blood from the femoral vein and the brachial artery during leg exercise The procedure has earlier permitted an evaluation of the functional impairment in patients with arterial circulatory insufficiency in the legs (Carlson and Pernow 1959 1962

During leg exercise the O_2 saturation of the femoral venous blood evidently falls almost linearly with successive rise in work intensity This decline is presumably only to a minor degree due to an increasing extraction of O_2 from the muscular blood Of greater importance seems to be the fact that the fraction of blood from non muscular tissue in the femoral venous blood probably decreases during exercise as a consequence of the marked increase in blood flow through the active muscles The extremely low O_2 saturation of the femoral venous blood during exercise of very high intensity indicates that at this level of work the femoral vein contains almost exclusively blood from the active muscles A gradually increased distribution of the peripheral blood flow to the working tissue thus appears to be an important factor in the ability to perform exercise of high intensity Disturbances of this mechanism for redistribution leading to a low physical working capacity have been demonstrated in individuals with vasoregulatory asthenia (Carlson Pernow and Zetterquist 1962) The almost complete peripheral O_2 extraction found in some cases in this series further illustrates that normally the O_2 transporting capacity is a limiting factor for the physical working capacity

It is earlier well documented that the formation of lactate is rapidly increased when the *exercise reaches its maximum A linear relationship was found between the log* lactate and work intensity which agrees with earlier findings (Strandell 1964) There is also a positive correlation between the aerobic and anaerobic components in the total muscular metabolism during exercise as indicated by Fig 6

The progressive and mainly parallel increase of the arterial concentrations of total lactate and XL during heavy work with increasing load found in this study is in agreement with earlier results (Carlson and Pernow 1962 Knuttgen 1962 and Strandell 1964) A steeper slope was however found for the regression of log venous XL than of log venous lactate on heart rate This finding may support the concept that XL is more closely correlated to the rate of anaerobic metabolism than lactate (Huckabee 1958) The absence of a similar difference in the increase of arterial concentrations of XL and lactate during work might be explained partly by a dilution effect partly by a higher degree of influence from lactate and pyruvate catabolism in other tissues on these values

Immediately after exercise the O_2 saturation of the venous blood rises very rapidly to values significantly higher than the initial level in the same body position before exercise. This agrees with the findings of Kramer Obal and Quensel (1939) in dogs They found that the O_2 consumption of the muscles after an initial peak immediately after the end of exercise fell very quickly and within no more than 30 sec after completion of exercise was about 50 per cent of that measured at the end of the exercise The post exercise hyperemia on the other hand is normalized very slowly after exercise of high intensity and long duration (Kramer *et al* 1939 Ganz *et al* 1964 Pernow and Wahren unpublished observation)

Dunér (1959) reported that the physical working capacity of a subject using one leg was about 75 per cent of that if two legs were used O_2 uptake at the same relative working intensity was appreciably less in one leg exercise Arterial lactate concentration increased more quickly during one leg exercise Dunér stated that in exercise involving large groups of muscles the limiting factor is the ability to supply O_2 to the muscle rather than the quantity of muscular mass participating The results obtained in the present study appear to corroborate this assertion When the motivation is strong it is possible in one leg exercise to attain virtually the same heart rate and O_2 uptake

as would be attained in two leg exercise (cases 1 and 4 in Fig 7) Since apparently both the cardiac output and the peripheral utilization of O_2 is the same whether a certain work is performed with one or both legs it might be assumed that the blood flow through the exercising muscles is much larger at the one leg than the two leg work However in spite of this the utilization of muscle glycogen as a source of energy is evidently more pronounced during the one leg exercise as indicated by the higher lactate level of the femoral venous blood

Obviously the reproducibility of the metabolic data presented in this series of papers is very good as seen in Fig 8—9 Regardless of whether the examination was repeated after a few hours of rest of a year later when the individual was in a comparable state of training the variations in a v O_2 difference pyruvate concentration and pH of the femoral venous blood at comparable absolute working intensity were very slight The variations in lactate concentration were somewhat greater

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Studies on the Elimination of Exogenous Lipids from the Blood Stream

The Kinetics of the Elimination of a Fat Emulsion Studied by a Constant Infusion Technique in Man

By

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Abstract

HALLBERG D *Studies on the elimination of exogenous lipids from the blood stream The kinetics of the elimination of a fat emulsion studied by a constant infusion technique in man* Acta physiol scand 1965 64 299—305 — The earlier described kinetic principle for the elimination of a fat emulsion (Intralipid®) from the circulation studied after single injections was confirmed in this study using a constant infusion technique A primer dose of the fat emulsion followed by a constant infusion was given The plasma triglyceride (TG) level was followed The emulsion TG fraction in plasma was separated from endogenous plasma TG with a polyvinylpyrrolidone density gradient At low infusion rates there was a constant TG level in the top fraction of the gradient containing the emulsion TG indicating a first order removal At high infusion rates there was a linearly increasing TG concentration in the top fraction indicating a zero order removal. The data confirm equivocally the kinetic principle described earlier which consists of a maximal elimination capacity (zero order kinetics) at high concentrations and an exponential elimination rate (first order kinetics) at low concentrations During the elimination of the emulsion TG fraction from the circulation changes were noted in the concentrations of the other TG fractions in plasma Some data on these changes are also presented

The elimination curve for chylomicrons and fat emulsions injected into the blood stream has been studied earlier by several authors who described the elimination to be either single exponential with a rate inversely proportional to the initial concentration (the given dose) (French and Morris 1957 Edgren 1960) or complex (French Morris and Robinson 1956 Fredrickson McCollester and Ono 1958) Controversially to this Carlson and Hallberg (1963) found in the dog that the elimination after single injections could be characterized in a simple mathematical form Above a so-called critical concentration (C mmole/l plasma) there was a linear (zero order) elimination rate (K_1 mmol l plasma min) and below this concentration there was a single exponential

TABLE I Data from constant i v infusions of fat emulsion in man Calculations are made on the triglyceride concentration in the top fraction after PVP density gradient separation of plasma See Table II for notation

Expt no	Pa	V	Period	t	C_L	C	K_I	K	C
1	2 41	116	I	70	0 43			0 13	
			IV	70				0 13	
2	3 05	146	I	50	0 75			0 07	
		292	II	50		0 0018	0 11		
									1 58
		438	III	50		0 0475	0 11		
			IV	30			0 12		
3	3 35 (P=3 3)	140	I	105		0 0081	0 04		
			IV	3ɔ			0 04		
4	3 27 (P=3 2)	53	I	52	0 14			0 13	
		132	II	50	0 23			0 14	
		264	III	49	0 71			0 12	
			IV	30				0 10	
5	1 98	25	I	52					
		50	II	52	0 23			0 13	0 23
		75	III	50		0 0096	0 03		
			IV	50			0 03	0 11	0 7
6	2 71 (P=2 8)	120	I	182		0 0110	0 03		
			IV	40			0 03		

Nondetectable concentration

(first order) elimination rate (K_2/min) The formula presented was K C = K_I This kind of kinetic was also valid for chylomicrons in dogs (Carlson and Hallberg 1963) and for artificial fat emulsion given as single injections to man (Hallberg 1964)

However to be able to study the human elimination curve it was necessary to separate fat emulsion from endogenous plasma triglycerides (TG) This could be done using a density gradient method with polyvinylpyrrolidone (PVP) described by Gordis in 1962 In this gradient the fat emulsion rises to the top of the tube and endogenous plasma TG separates into two fractions which remain in the lower part of the tube called middle and bottom fractions (Hallberg 1964) *The object of the present investigation was to* study further the described kinetic principle in man be means of a constant infusion technique

Materials and methods

Subjects Male and female patients in apparently good general condition between 20 and 40 years old hospitalized for minor disorders (hernia hemorrho[illegible]ds etc) were used for th[illegible]s investigation After an overnight fast (about 15 hrs) the studies were performed in the morning with the patients resting in bed during the experiment

TABLE II Table of notation

Symbols	Definitions	Units
\	Amount of triglyceride infused as a 10 emulsion per unit time	mg/min
I II III	Period of infusion	
IV	Post infusion period	
t	Length of infusion period	min
C	Critical concentration the concentration of TG in plasma where zero order kinetic changes to first order kinetic	mmole/l
C	Increase in plasma TG concentration during constant infusion of fat e nulsion	mmole/l/mi 1
C_L	Constant level of plasma TG concentration during co 1 stant infusion of fat emulsion	mmole/l
K_1	Constant for zero order kinetic	mmole/l/min
K	Constant for first order kinetic	/min
Pa	Assumed plasma volume (43 ml/kg)	l
P	Determi 1ed plasma volume (Evans blue method)	l
M	Mean molecular weight for the triglycerides of Intralipid®	

Constant infusion A Beckman solution metering pump adjustable to 0—5 ml/min was used for the constant infusions The pump was repeatedly cal brated during the study Immediately before the start of all i v infus ons a primer dose of 0 1 g triglycerides per kg b w as fat emulsion was g ven i v during less than two minutes (Primer dose in case no 6 was 0 05 g/kg) This dose was repeated each time the speed of the infusion was increased

S x subjects v ere divided into two groups A and B with 3 persons in each group Group A r ceived infusions during one period and group B during 3 consecutive periods with an increasing amount of fat for each period The durat on of each period is hown in Table I (range 49—18? min) The concentration of pla ma TG was follo ed before during and after the infus ons After fractionat on of the plasma TG in the PVP gradient each fract on was analysed for the TG co icentration

Procedures All nfus ons we e given into a cubital vein Samples of blood were withdrawn from a permanent needle in a vein in the other arm and collected nto chilled heparini ed tubes which were stored in ice water not longer than 4 hrs befor extraction All constant infus ons of the fat emulsion were performed with Intral pid® 10 (Schuberth and Wretl nd 1961) All rap d injections (primer doses) we made w th Intral p d® 20 (except n case no 6 where the 10 emulsion as used) The volume of distribut on of injected l p ds was assumed to be equal with the plasma olume (B erman and Hamlin 196.) Pla ma volume was assumed to be 43 ml per kg b w In some instances th s figure was checked with the Evans blue method and found accurate The mean molecula weight of the TG in Intralip d® was determined by comparison with a standard of tripalm tin The commercial tripalm t n reagent grade was recryst ll zed three t mes purified on a silic c acid ollumn and the purity was controlled with

The en uls on was kindly supplied by A B Vitrum Stockholm Swed n

thin layer chromatography The TG content of the emulsion was given by the manufacturer The mean molecular weight determined from the glyceride glycerol content was found to be 885 The mean m w of soya bean oil was 874 calculated from the fatty acid composition given by Wretlind (1963) and if 5% of undetermined fatty acids was considered to be stearic acid.

Analytical methods All TG determinations were made by the method of Carlson (1963) Triplicate determinations were carried out on each extract Every other plasma sample was extracted in duplicate

Fractionation of plasma TG were made by a modification of the density gradient method with PVP described by Gordis (1962) (Hallberg 1964) In the present study the recovery of TG after separation was 107 ± 10% (SD) The errors of the analyses were otherwise the same as those found earlier (Hallberg 1964)

Calculations

Calculations were made only for the top fraction TG Zero order removal (K_1 mmole/l min) was considered to exist when the curve increased linearly during infusion or fell linearly after infusion (zero order kinetic) (Daniels 1951) First order removal (K_2/min) was considered to exist when the curve was on a constant level during the infusion or fell exponentially (first order kinetic) after the termination of the infusion (Daniels 1951) Rate constants (K_1 and K_2) were determined by calculations

$(K_1 + C)$ P V = V (during linearly increasing TG concentration)

K_2 C_L P V = V (at constant TG concentration)

(See Table II for notations)

Rate constants for period IV were determined from the slope of the curves on either linear or semilogarithmic scales (Hallberg 1965) and they are given in Table I The so-called critical concentration (C mmole/l) is defined as the concentration where the fractional removal rate equals the maximal removal rate K_2 C = K_1

Results

Group A (One infusion period) In the *top fraction* two subjects showed a linearly increasing TG concentration and one showed a constant TG level during the infusions (Fig 1) After the termination of the infusions the TG concentration fell in all the subjects The linear change indicates a zero order reaction for the removal whereas the constant level during the infusion indicates a first order reaction

The *middle fraction* TG concentration increased for all 3 subjects In the two with the lowest concentrations the middle fraction increased up to a constant level After the termination of the infusions one of the curves fell exponentially one constantly increasing curve levelled off and the third seemed to fall after a delay of 15 min (Fig 1)

The *bottom fraction* TG concentration showed a slow increase during the infusions After the termination of the infusions the curves did not show any consistent changes (Fig 1)

Group B (3 consecutive infusion periods) The *top fraction* TG concentration in group B showed different levels during the different periods of infusions (Fig 2) During the first period (I) when the smallest amount of fat was infused there was a constant TG level During the second (II) period there were constant levels for two curves and a small increase in the third curve During the third (III) period there was a linear increase in concentration for two subjects and one had a constant level During the post infusion period (IV) all curves declined The linear change indicates a zero order reaction and the constant level during the infusion indicates a first order reaction

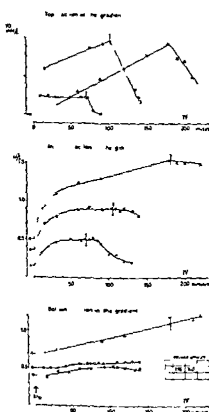


Fig 1

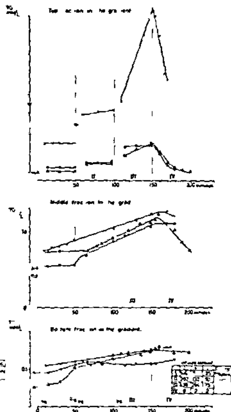


Fig 2

Fig 1 (left) Triglyceride concentration (mmoles/l) in plasma from 3 subjects (no 1 3 and 6) given constant i v infusions of fat emulsion (Intralipid®) Plasma triglycerides were fractionated into 3 fractions (top middle and bottom) in PVP gradient tubes (see methods) Arrow indicate the primer dose (0 1 g fat/kg in no 1 and 3 and 0 05 g fat/kg in no 6) and the start of infusions the vertical lines indicate the end of the infusions The table shows the amounts of fat infused per min I indicates the infusion period and IV the period after infusion.

Fig 2 (right) Triglyceride concentration (mmoles l) in plasma from 3 subjects (no 2 4 and 5) given constant i v infusions of fat emulsion (Intralipid®) during 3 periods (I II and III) Plasma triglycerides were fractionated into 3 fractions (top middle and bottom) in PVP gradient tubes (see methods) Arrows indicate primer doses at the beginning of each period (0 1 g fat/kg) The table shows the amounts of fat infused during each period (I II and III) IV indicates the period after the end of the infusions

The *middle fraction* TG showed a continuously increasing concentration during the infusion except during period I where two curves came up to a constant level After the infusion the concentrations decrease or remained unchanged

The *bottom fraction* TG in this group showed an almost continuously increasing concentration during the infusions except in one case where it came up to a constant level during periods II III and IV

Rate constants The rate constants for the elimination of top fraction TG were calculated according to the formulas described and are shown in Table I

Within each experiment there was good agreement for the calculated values of the constants between different periods

All the elimination constants calculated during the infusion were of the same size as the constants determined on the graphs for the postinfusion period The constant for maximum removal capacity varied between 0 03 mmole/l/min and 0 12 mmole/l/min and the constant for the fractional removal rate varied between 0 07 and 0 14 per min

Discussion

As it was difficult to know the critical concentration at the beginning of each experiment the given doses were not of the correct magnitude to obtain maximum information from each experiment However if the results are looked upon as a whole the kinetic principle for the top fraction can be judged and some information about the kinetics for the middle fraction TG be obtained

Top fraction TG These experiments confirm the principle of the kinetics described earlier for the elimination of the used fat emulsion from the blood (Carlson and Hallberg 1963 Hallberg 1964) There exists a maximal capacity (zero order kinetic) for the removal of artifical fat emulsion from the blood stream in man Below a critical concentration the rate of removal is exponential This kinetic principle was earlier shown to be valid for chylomicrons in the dog (Carlson and Hallberg 1963) The kinetic principle was confirmed by the experiments with several infusion periods and with different amounts of emulsion infused The elimination constants were of the same size both during and after the infusion and independent of the TG concentration when the reaction orders were the same

It was earlier observed that during the elimination of the fat emulsion in man changes occurred in the concentration of the endogenous plasma TG found in the middle and bottom fractions in the PVP gradient (Hallberg 1964) so was also the case in this investigation The literature available at present contains no information about the kinetics of these plasma lipid fractions in the PVP gradient This investigation was not designed to follow these fractions in detail The results may however be commented upon

Middle fraction TG In group-A experiments (one infusion period) there were two curves showing a steady increase of concentration up to a constant level It can be assumed that during the interval of time when the concentration remained at a constant level the amounts of middle fraction TG entering and leaving the plasma compartment were equal In one of the curves (no 1) which was followed long enough after the end of the infusion the concentration decreased exponentially (with a half life of 32 min [$K = 0\ 022$]) *This suggests that the level was regulated by a concentration-dependent* process (first order kinetic) When the middle fraction TG concentration was at a constant level during the infusion there was a delay of about 15 min at the end of the infusion, until the concentration started to decrease (no 1 Fig 1) This suggests that the middle fraction TG concentration is not directly dependent upon the top fraction TG concentration.

In group-B (3 infusion periods) there were also two middle fraction curves coming up to a constant level *during the first period (first order kinetic)* During two consecutive

periods the concentration seemed to increase in a linear way (zero order kinetic) After the end of the infusion one of the curves (no 5) fell in an apparently linear way This suggests that the kinetic for the middle fraction TG may be the same as that for the fat emulsion. This suggestion has been the subject of further studies (Hallberg 1965)

In group-B experiments there was a continuously increasing TG concentration in the middle fraction after the first period in 5 of 6 periods This kind of increas argues against contamination of fat emulsion TG from the repeated primer doses and for the opinion that this fraction is recirculating TG

Bottom fraction TG The TG concentration in this fraction increased slowly in all the cases There were no definite trends in the curves and no conclusions concerning the kinetics for this plasma fraction can be drawn from these experiments The increase per minute was of the same magnitude as that observed in the bottom fraction after centrifugation of plasma at 48 6 × 10 g min after single injection of fat emulsion (Hallberg 1964)

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Studies on the Elimination of Exogenous Lipids from the Blood Stream

The Kinetics of the Elimination of a Fat Emulsion Studied by Single Injection Technique in Man

By

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Abstract

Hallberg D *Studies on the elimination of exogenous lipids from the blood stream The kinetics of the elimination of a fat emulsion studied by single injection technique in man* Acta physiol s and 1965 64 306—313 — The kinetics of the elimination of a fat emulsion (Intralipid®) from the blood stream was studied after single injections to 10 fasting individuals Plasma triglycerides (TG) were fractionated by means of polyvinylpyrrolidone density gradients into three fractions The emulsion TG was found in the top fraction of the gradient and endogenous plasma TG separated into two fractions which were found in the lower part of the density gradient The kinetics of the emulsion TG fraction were composed of a zero order elimination rate at high TG concentrations and a first order elimination rate at low TG concentrations Intra individual variations were only observed in the zero order part of the kinetics the possible causes of these variations are discussed Changes in concentration of the lipids found in the lower part of the density gradient were observed during the elimination of the emulsion TG from the circulation The possibility that the kinetic principle for one of these fractions (middle fraction) is the same as that for the fat emulsion is discussed

The elimination curve for fat emulsions leaving the blood stream after intravenous injection has been shown to be composed of 2 parts representing a zero order reaction at high triglyceride (TG) concentrations and a first order reaction at low TG concentrations The rate of the elimination is determined by one constant for each order of reaction This was shown to be valid in dogs (Carlson and Hallberg 1963) after single injections of a fat emulsion The kinetic of fat emulsion leaving the blood stream in man could only be determined after separation of emulsion from endogenous plasma TG by a density gradient method with polyvinylpyrrolidone (PVP) described by Gordis in 1962 (Hallberg 1964) With this method it was shown that the kinetic principle in man for fat emulsion leaving the blood stream was the same as that in dogs after single

TABLE I Rate constants for the removal of injected fat emulsion from plasma in man Calculations are made on the curve for the TG concentration in the top fraction after PVP density gradient separation of plasma

Case no	Dose g/kg	K_1[1] mmole/l/min	T 1/2 min	K [2] /min	C[3] mmole/l
1	0 3	(>0 40)	6	0 12	(>4 0)
2	0 3	0 11	11	0 06	1 75
3	0 3	0 08	18	0 04	2 0
4	0 3	(>0 10)	17	0 04	(>2 5)
5	0 3	0 09	10	0 07	1 30

K_1 = Maximal removal rate i e rate for linear phase Figures within parentheses are calculated from $C = \frac{K_1}{K}$

K = Fractional removal rate i e rate for exponential phase

[3] C = Critical concentration glyceride concentration where linear phase changes into exponential phase Figures in parentheses are taken from the highest value in the exponential part of the curve

TABLE II Rate constants on different days for the removal of injected fat emulsion from plasma in man Calculations are made on the curve for the TG concentration in the top fraction after PVP density gradient separation of plasma

Case no	Dose g/kg	K mmole/l/min	T 1/2 min	K /min	C mmole/l
6 A	0 3	0 13	13	0 05	2 60
B	0 3	(>0 15)	13	0 05	(>3 0)
7 A	0 3	0 10	9	0 08	1 3
B	0 3	(>0 37)	9	0 08	(>4 0)
C	0 3	0 06	9	0 08	0 78
8 A	0 3	(>0 06)	17	0 04	(>1 5)
B	0 5	0 07	17	0 04	1 8
9 A	0 2	(>0 07)	12	0 06	(>1 2)
B	0 5	(>0 17)	12	0 06	(>3 0)
10 A	0 5	0 06	17	0 04	1 54
B	0 2	(0 04)	19	0 04	(>1 0)

K = Maximal removal rate i e rate for linear phase Figures within parentheses are calculated from $C = \frac{K}{K}$

K = Fractional removal rate i e rate for exponential phase

C = Critical concentration glyceride concentration where linear phase changes into exponential phase Figures in parentheses are taken from the highest value of the exponential part of the curve

injections The kinetic was also confirmed in man by a technique with constant i v infusion of the fat emulsion (Hallberg 1965)

Bierman Gordis and Hamlin (1962) demonstrated with the PVP gradient method that during alimentary lipaemia a recirculating lipid fraction called secondary particles was present This was found in the middle fraction of the gradient tube During the elimination of the fat emulsion both after single injections and during constant infusions it was observed that the lipid concentration in the endogenous plasma TG fractions in the PVP gradient changed (Hallberg 1964 1965) This suggests a recirculation of the emulsion TG into the blood stream It was also observed that there were inter as well as intra individual variations in the elimination curves The aim of the present work was to extend these studies with single i v injection of a fat emulsion Data for the recirculating TG fractions observed in the present work will also be discussed

Material and methods

Procedures 16 studies were made in 10 subjects Some individuals were investigated 2 or 3 times with intervals of at least 3 days usually 7 days After an overnight fast individuals in good general conditions were given varying doses of the fat emulsion (Intralipid® 20)[1] i v into an arm vein The amounts of given fat emulsion are set out in Table I and II One individual had fasted longer as stated below The injection time was less than 2 min Blood samples were drawn at intervals from a permanent needle in a vein in the other arm The blood was collected in chilled heparinized tubes and stored in ice water not longer than 4 hrs before extraction After the test all samples were centrifuged to separate off the plasma at 400 g min (g min according to *Dole* and *Hamlin* 1962)

The TG concentration was determined on plasma before and on the three fractions after separation of plasma in PVP density gradients

Analytical methods All TG determinations were made by *Carlson s* (1963) method The mean of triple determinations on each extract are given Many of the plasmas were extracted in duplicate The fractionation of plasma in PVP gradients was made by a quantitative modification of a method described by *Gordis* in 1962 (*Hallberg* 1964) The PVP gradient separates lipaemic plasma into three fractions 1 *Top fraction* in the gradient tube this fraction contains the emulsion TG (*Hallberg* 1964) 2 *Middle fraction* in the gradient tube this fraction contains a part of the endogenous lipids 3 *Bottom fraction* in the gradient tube this fraction contains the bulk of endogenous lipids The different fractions were separated with a tube slicer and by puncture of the plastic tubes with a needle The errors of the analyses have been given earlier (*Hallberg* 1964) Samples with recovery out of 103 ± 20 were excluded

Calculations and definitions All results were plotted on graphs TG concentration versus time on linear and semilog scales The slope of a linear curve on a linear scale is designated K (mmole/l plasma/min) (zero order kinetic) The slope of a linear curve on a semilog scale is called K_2 (per min) (exponential or first order kinetic) The concentration where the curves change order of reaction is called the critical concentration (C mmole/l) and can be determined by the formula K_2 $C = K$ In those curves showing only an exponential elimination rate the K was estimated from the highest concentration of the exponential part of the curve These estimated figures give a minimum value of C and thus also a minimum value of K

The curves for middle and bottom fraction TG are in the figures drawn with interrupted lines

Results

The elimination curves were all complex as judged from the curves for the concentration of total TG in plasma as described earlier (Hallberg 1964) Results from each of the three fractions derived by separation of plasma in the PVP gradient will be presented here

[1] The emulsion (Schuberth and Wretlind 1961) was kindly supplied by A B Vitrum Stockholm

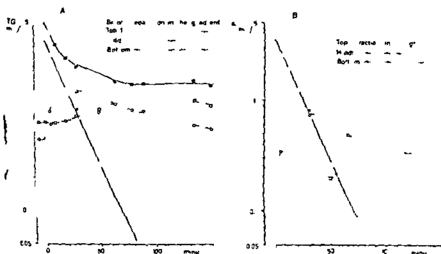


Fig 1 Elimination from the blood stream of intravenously injected fat emulsion (0 3 g fat/kg case no 8A) A The triglyceride concentration in plasma before and after separation in PVP gradients into three fractions in semilogarithmic scale B Changes from preinjection value in triglyceride concentration in the three gradient fractions in fig A

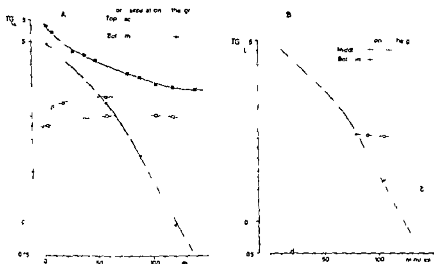


Fig 2 Elimination from the blood stream of intravenously injected fat emulsion (0 5 g fat/kg case no 8B) A The triglyceride concentration in plasma before and after separation in PVP gradients into three fractions in semilogarithmic scale B Changes from preinjection value in triglyceride concentration in the three gradient fractions in fig A Semilogarithmic scale

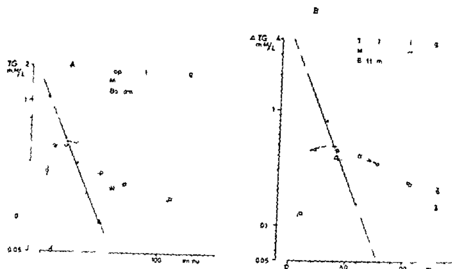


Fig. 3. Elimination from the blood stream of intravenously injected fat emulsion (A 0.2 g fat/kg case no. 9A, B 0.5 g fat/kg case no. 9B). Changes from preinjection value in triglyceride concentration of plasma after separation into three fractions in PVP gradients. Semilogarithmic scale.

Top fraction. This fraction in the gradient represents the fat emulsion TG in plasma (Hallberg 1964). The elimination curve was composed of a linear part at high TG concentrations and an exponential part at low TG concentrations 8 times in 7 subjects as described earlier (Hallberg 1964). All the other times the elimination curves were exponential. The composite curves confirmed the kinetic principle earlier described with a zero order kinetic at high concentrations and a first order kinetic at low concentrations. The elimination constants were calculated in each case and are set out in Table I and II. The size of K_1 varied between 0.06 and > 0.40 mmole/l plasma/min and K_2 between 0.04 and 0.12 per min. There were inter- as well as intra-individual variations in the curves. Intra-individual variations were found in the zero order part (K_1) of the elimination curves in case no. 7. The first order parts (K_2) were identical within each of the 5 subjects investigated more than once (Table II).

Case no. 8 with a single exponential elimination curve with the smaller dose was reinvestigated with a larger dose and then the curve showed both orders of elimination (Fig. 1A and 2A). K_2 was the same (0.04/min) in both studies.

Case no. 7 was investigated 3 times with the same dose and underwent a cholecystectomy in the first interval and had no food for the first 2 postoperative days. The slopes of the exponential part (K_2) of the curves were identical on these 3 occasions. However, the constant for the zero order part of the curve (K_1) showed variations between 0.06 mmole/l/min and up to more than 0.32 mmole/l/min, see Table II.

Middle fraction. This fraction in the PVP gradient represents a part of the endogenous plasma lipids. During the elimination of the fat emulsion the concentration of the middle fraction TG was first increasing (Fig. 1—3), then in some cases remaining at a plateau (e.g. Fig. 2) and thereafter decreasing. In order to estimate changes in middle

fraction TG concentration during disappearance of TG in the top fraction the middle fraction concentrations minus the fasting concentration were plotted against time on semilogarithmic scales for all curves (e g Fig 1B 2B 3) The decreasing parts of 11 curves so constructed were compatible with an exponential decay with a half life between 20 and 40 min The other 5 subjects were either not followed long enough to reach the decreasing part of the curve or the points did not permit a judgement of the shape of the curves The curve for the top fraction TG concentration crossed the curve for the increase in middle fraction TG concentration fairly well at its peak (Fig 1B 3A) The response in the middle fraction TG to different doses of fat emulsion is illustrated in Fig 1 and 2 (case no 8) and in Fig 3 (case no 9) The middle fraction TG seem in both cases to increase up to the same level with the different doses but with the higher doses the middle fraction remains longer at this level (Figs 2B and 3B)

Bottom fraction This fraction in the PVP gradient contains endogenous lipoproteins The injection of fat emulsion caused increases in TG concentration in all cases These changes were always smaller than those in the middle fraction and the peak if present appeared at a later time At the end of the experiment the concentration was decreasing in most cases Whether this decrease is exponential or not cannot be determined from the present experiments The inter as well as intra individual variations were not so marked as in the top and middle fractions After subtraction of the fasting concentration the peak of the increase coincided in most cases with the decreasing part of the curve for the increase of the middle fraction In two instances however the maximal increases did not coincide with the middle fraction (Figs 2B and 3B)

Discussion

Elimination of the fat emulsion (top fraction)

The kinetic principle described earlier for elimination of a fat emulsion from the circulation (Carlson and Hallberg 1963 Hallberg 1964 1965) was verified in this investigation The interpretation of curves showing only exponential elimination rates is probably that the given amount of fat was too small for the plasma concentration of TG to reach the critical concentration where the maximal elimination rate acts (zero order kinetic) The validity of this explanation is illustrated by case no 8 showing both types of curves with different doses (Fig 1 and 2)

There were inter as well as intra individual variations in the elimination rates The intra individual variations were only found in the maximal removal capacities (K_1 zero order part of the kinetics) The slopes of the exponential part of the curves (K_2) did not vary from time to time in cases investigated more than once One case (no 7) showed an increased removal capacity 2 days after an operation for gallstones and unchanged fractional removal rate The cause of the increase in the removal capacity may be either the trauma or e g the difference in nutritional state As the other cases also showed variations without trauma the nutritional state may be of importance in the regulation of K_1

Recirculating lipids (middle and bottom fraction)

The relation between the changes in lipid concentrations in the three gradient fractions seems in most curves to be close to a precursor product relationship as described by Zilversmit *et al* 1943 The curves fulfil the requirements that R ggs (1963) states for

such a relationship Each successive peak is lower than the last the precursor curve crosses the product curve at maximum the precursor maximum comes sooner than the product maximum There are however some curves that do not fit into this system (Fig 2B and 3B) The mathematical precursor product equation by Zilversmit does not take a zero order kinetic for the precursor into consideration

Let us assume that the fat emulsion passes from the circulation into a pool (the liver?) which transforms it into lipids that reappear in the circulation and possess properties similar to those of the middle fraction lipids Then zero order kinetic for the emulsion in plasma will act as a constant infusion into the pool and the product from that pool will act as a constant infusion into the circulation This assumption is supported by experiments with constant infusion of fat emulsion (Hallberg 1965) In those studies it was observed that at the end of the infusion there was a delay until the middle fraction decayed in concentration The delay can be explained by the presence of a pool between the top fraction TG and the middle fraction TG Such a pool can of course also explain deviations from the ideal precursor product relationship

The possibility that the kinetic principle for middle fraction lipids in plasma is the same as that for the emulsion is supported by other observations during the experiments with constant infusions At low infusion rate of the fat emulsion the middle fraction remained at a constant level (first order reaction) and at the end of the infusion after the delay it seemed to decrease exponentially (first order reaction) At higher infusion rates the middle fraction increased linearly suggesting a zero order kinetic In the present study the shape of the middle fraction curves in Fig 1 2 and 3 supports these possibilities

The concentration of TG in the bottom fraction also changed during the elimination of the fat emulsion From the shape of the curve for the changes in concentration and according to the quoted statement by Riggs it seems that this lipid fraction contains recirculating material from the middle fraction From the present study it is impossible to draw any further conclusions concerning the kinetic principles governing this fraction

The earlier concept that the elimination rate for chylomicrons and fat emulsion is determined by the dose (French and Morris 1957 Edgren 1960 Bierman and Hamlin 1962) may have a biological explanation by combinations of zero order and first order kinetics for precursor and product in different pools

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On the Regulation of Sweat Secretion in Exercise

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Abstract

Nielsen B and M Nielsen *On the regulation of sweat secretion in exercise* Acta physiol scand 1965 *64* 314—322 — With exception of the first few minutes of muscular exercise the sweat rate both in the work and the recovery period was found to follow closely the changes in deep esophageal and tympanic temperatures — *In the steady state of work of various intensities performed at a constant environmental temperature the mean skin temperature remained constant* whereas there was a linear relationship between sweat rate and internal temperatures measured as rectal esophageal and tympanic temperatures *In the steady state of work of constant intensity but at various environmental temperatures (5 C to 30 C) the internal temperatures (rectal esophageal and tympanic) remained constant* and the sweat rate *(150 to 750 g per hr) increased nearly linearly with increasing mean skin temperature* The mean skin temperature was however even at 30 C amb temp lower than that at which sweating was elicited in rest — *Elimination of the temperature gradient along the esophagus by breathing air saturated* with water vapor at body temperature had no effect on either sweat rate or tympanic and other internal temperatures The effect of vigorous cooling of the neck over the carotid arteries was only small

During muscular exercise the body temperature increases to a new level the height of which is dependent on the intensity of work (kpm per min or O_2 uptake per min) At constant work intensity the increased body temperature is maintained at the same level independent of wide variations in environmental temperature (M Nielsen 1938) The large changes in heat loss by radiation and convection called forth by the variations in environmental temperatures were exactly counterbalanced by changes in evaporation of sweat These results indicate that the increase of body temperature in exercise is not due to a failure of heat regulation but is due to a change in the setting of the regulatory mechanism This view is supported by experiments of Winslow and Gagge 1941 Robinson 1949 Wyndham *et al* 1952 and Lind 1963 The temperature in the deep part of the esophagus considered to be a good index of the aortic blood temperature showed the same independence of environmental temperature as did the rectal temperature (B Nielsen and M Nielsen 1962 Minard and Copman 1963)

Recently Benzinger (1959 and 1960) found that changes in sweat secretion caused by variations in the environmental temperature by work or by combination of both were very closely correlated to changes in the temperature measured at the tympanic membrane He proposed that changes in sweat secretion exclusively depend on the temperature of the heat loss centre in the anterior hypothalamus He considered the temperature of the tympanic membrane a good index of the hypothalamic temperature as these two organs both are supplied with blood from the internal carotid artery Benzinger s theory seems to be in contrast to the above mentioned experiments in which large variations in sweat production were found during work of constant intensity performed at varying external temperatures in spite of the fact that the internal (rectal or esophageal) temperature remained constant

In the experiments presented in this report we have studied the correlation between sweat secretion and rectal esophageal tympanic and skin temperature in rest and during work The work experiments were performed both with constant work intensity at varied envionmental temperature and with varied work intensity at constant en vironmental temperature Further we have attempted to diminish or to increase the cooling of the blood which possibly occurs on its passage from the trunk to the head

Methods

The rectal temperature was measu ed at 4 different depths and the esophageal temperature just above the diaphragm as described by B Nielsen and M Nielsen (1962) The tympanic temperature was mea ured by means of a thermocouple consisting of a 0 08 mm thick copper wire and a 0 09 mm thick constantan wire the junction of which was soldered to a small s lverplate (1 5 mm in diameter and 0 4 mm thick) The 15 cm long wires were rolled up nto a 3 cm long spi al and mounted in a polyethylene tube from which about 2 mm of the spiral bearing the s lverplate pr truded The thin copper and constantan wires forming the thermojunction were isolated from cables contain ng 6 strands of copper or constantan False junctions between the thin wires and the cable were thus avo ded The polyethylene tube was wound with cotton so that after the insertion it fitted tightly to the auditory meatus The most reproducible results were obtained when the subject h mself brought the thermocouple in full contact with the tym panic membrane a couple of m nutes before each measurement By pulling the ea lobe upward the meatus was straightened while the thermocouple was mo ed the last fraction of a mm until the characterist c slightly painful sensation was felt

The skin temperature was measured either by a thermocouple o by a thermoelectric radiation receiver on 15 d fferent places (head 2 trunk 7 upper extremity 3 and lower extrem ty 3) The mean skin temperatu e was calculated by weigh ng the measurements in proportion to the size of the corresponding areas (Hardy and DuBois 1938) The sk n thermocouple was made by stretch ng a thin copper and a th n constantan wire over the end of a short hollow plastic cylinder (d ameter 1 9 m) so that they crossed on another in a thermojunction in the centre of the cylinder opening The cylinder wa furnished with a 15 cm long handle through which the connecting w res passed. W th this skin thermocouple a constant read ng could be obtained within a couple of seconds after the pparatus was placed aga nst the skin and the measure ments were practically unaff cted by small variations n the exerted p essure Further heat conduct on from the thermojunction was minimized because the adj cent wi es were n contact with the skin during the measurement

As rad ation rece ver was used a Moll thermop le (large surf ce thermopile E_{II} K pp and Zonen) The apparatus was nsulated with plastic foam enclosed n a covering of polished alu m nium from wh ch the reflector protruded 3 mm To protect the thermop l against a r cu rents the aperture of th reflecto wa co ered by a fil n of pliofil n (th ckness about 10 μ) The time of response was only 5 –7 sec and was only slightly n reased by use of the pliofilm The radiat on from the sk n was n a h series of measurement (head trunk etc) compared with the rad ation fron tw bla k bod es at temperatures a few degrees above respecti ely below the skin temperatu e The rad omet was prov ded w th 5 small plast c pins by means of

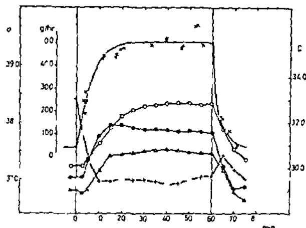


Fig 1 The rate of weight loss (×) and the average of the rectal temperatures (○) deep esophageal temperatures (●) tympanic temperatures (△) and mean skin temperatures (+) before during and after 60 min of work at an intensity of 900 kpm per min (O_2 uptake 2 1 l O per min) 5 expts Environmental temperature 20 C Forced air movement during and after the work period Subject H N

which the reflector could be held 2 to 3 mm above the skin without direct contact

The experiments were performed on a Krogh bicycle ergometer in which the saddle had been replaced by an armchair The weight loss was measured continuously during and after the work period by means of the Krogh balance (Krogh and Trolle 1936) in which the bicycle ergometer was suspended as described by M Nielsen 1938 The error on the weight loss determinations was less than 5 g during work The sweat secretion was determined from the total weight loss by substracting the weight loss due to the respiratory gas exchange and water loss The respiratory gas exchange was determined by the Douglas bag method In most experiments an electric fan directed towards the subject and producing an air movement of about 0 5—1 m/sec in chest height was used

Experiments in which the subject breathed air saturated with water vapor were carried out as described by B Nielsen and M Nielsen (1962) Cooling of the carotid blood was attempted by placing copper capsules which could be perfused with saline of 0 C on the neck over the carotid arteries The capsules were about 5 cm long and 2 cm wide and with rounded edges

The experiments were carried out in a climatic chamber (cf B Nielsen and M Nielsen 1962) The subjects were young male students (O A 21 years 77 kg 183 cm DuBois area 1 98 m aerobic work capacity about 4 4 l O /min H N 22 years 59 kg 174 cm DuBois area 1 71 m aerobic work capacity about 2 5 l O_2/min J V 24 years 78 kg 178 cm DuBois area 1 96 m) On entering the climatic chamber the subjects were placed in the ergometer chair and after 15—20 min of rest the initial measurements were made Immediately after that work was started In the rest experiments the subjects were lying on a bed of rubber bands fastened to a wooden frame suspended from the balance and usually a preliminary period of 60 min preceded the experimental period

Results

Fig 1 shows the rate of weight loss (g per hr) and the averaged rectal esophageal tympanic and mean skin temperatures from 5 expts on H N with a work intensity of 900 kpm/min (O uptake 2 1 l/min) and an environmental temperature of 20 C In Fig 2 is shown the rate of weight loss the rectal and the esophageal temperatures in 2 series of 5 expts each with the same work intensity on subjects O A one series performed at 20 C and the other at 37 5 C environmental temperature It can be seen from Fig 1 that the changes in tympanic temperature and in esophageal temperature both at the onset and at the end of work occur faster than the changes in rectal temperature In the work period the tympanic temperature level is lower than the

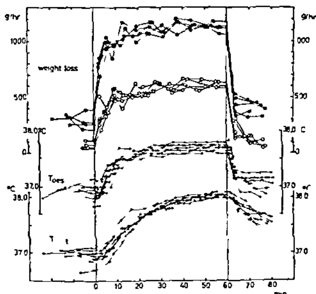


Fig 2 The rate of weight loss the esophageal and the rectal temperatures before during and after 60 min of work at an intensity of 900 kpm per min 5 expts at 20 C environmental temperature (○) and at 37 5 C (●)
Subject O A

esophageal temperature level which again is lower than the rectal temperature level The mean skin temperature falls considerably at the beginning of the work partly due to the air movements caused by the start of the electrical fan At the end of work the mean skin temperature again increases somewhat in spite of the continued forced air movement. The rate of weight loss begins to increase immediately at the start of work whereas the increase of the internal temperatures both at 20 C and at 37 5 C environmental temperatures are 1 5 to 2 min delayed After this initial period the changes in the rate of weight loss seem to run in parallel to the changes in the esophageal and tympanic temperatures both during the rest of the work period and in the recovery period. This time-relation between changes in rate of weight loss and changes in internal temperatures is found both in the experiments at 20 C and 37 5 C environmental temperature in spite of the large differences in rates of weight loss existing at the two temperatures (Fig 2)

In experiments in which the work intensity varied from 540 to 1 440 kpm per min at a constant environmental temperature of about 20 C, the rate of sweat secretion increases linearly with both rectal, esophageal and tympanic temperature (fig 3) The slope of the curve relating sweat secretion to rectal temperature is 30—40 per cent less than that of the other 2 curves This difference in the slopes can not be taken as evidence for a greater sensitivity of the sweating mechanism to changes in esophageal and tympanic temperature It is in agreement with the findings that the rectal temperature is relatively high because it is influenced by the warm blood from the working muscles (B Nielsen and M Nielsen 1962)

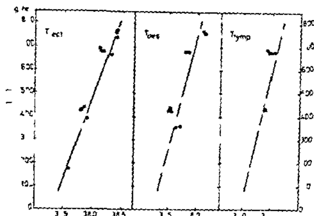


Fig 3 The steady state values of sweat rates at work intensities from 540 to 1 440 kpm per min plotted against the corresponding values of rectal deep esophageal and tympanic temperatures Environmental temperature 20 C
Subject O A

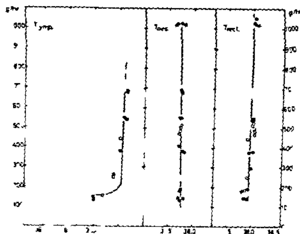
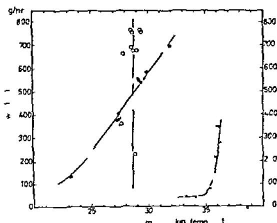


Fig 4 The steady state values of sweat rate plotted against the corresponding values of tympanic deep esophageal and rectal temperature Work intensity 900 kpm per min Environmental temperature from 5 C to 37 5 C Experiments with forced air movement (●) and without (○)
Subject O A

The rate of sweating in relation to rectal esophageal and tympanic temperatures in the steady state of work of 900 kpm per min at environmental temperatures between 5 C and 37 5 C is presented in Fig 4 The sweat loss varies between 125 and 1 070 g per hr while the rectal and the esophageal temperatures are constant. Also the tympanic temperature seems to be practically constant except in the lowest range of sweat secretion

Fig 5 shows the rate of sweat secretion in relation to mean skin temperatures under different conditions of rest and exercise The determinations are from the same experiments as those presented in Fig 3 and 4 With constant work intensity the sweat secretion increases nearly linearly with mean skin temperature Also during rest at increasing environmental temperature the sweat secretion is related to the mean skin temperature The skin temperature at which a certain rate of sweat secretion is elicited is however much higher in the resting than in the working condition In the experi-



F g 5 The steady state values of sweat rate plotted against the corresponding values of mean skin temperature (O) work intensity from 540 kpm per min to 1 440 kpm per min at constant environmental temperature of 20 C (●) constant work intensity (900 kpm per min) at environmental temperatures from 5 C to 30 C (×) rest experiments at environmental temperatures from 25 C to 44 C. Subject O A

TABLE I

A ɪ b temp C	S bject	kpm/min	Breathing water vapor T_R	T_{oes}	T_{tymp}	Weight loss g per 65 min	Forced air movement	Control T_R	T_{oes}	T_{tymp}	Weight loss g per 65 min	Forced air movement
10	OA	900	38 05	37 83	37 07	260	+	37 93	37 77	(–)	272	+
								37 80	37 75	(–)	278	+
10	HN	720	37 81	37 38	37 10	209	–	38 19	37 75	37 38	19J	–
			37 9?	37 60	37 20	260		38 13	37 82	37 39	259	–
20	OA	1 080	38 07	37 73	37 38	613	+	38 12	37 73	37 37	586	+
			38 09	37 78	37 65	614		38 27	37 9?	37 67	613	+
?0	HN	7?0	37 83	37 32	37 30	383	–	37 93	37 44	37 35	388	–
			37 90	37 48	37 45	395		37 98	37 58	37 47	378	–
			37 78	37 30	37 27	379	–	37 75	37 40	37 25	399	–

ments performed at constant environmental temperature the mean skin temperature remains constant at all work intensities (540 to 1 440 kpm per min)

In 8 expts a cooling effect from the respiratory passages on the arterial blood to the head was prevented by breathing air saturated with water vapo at body temperature (table I) Both the rate of weight loss and the internal temperatures are of the same magnitude as in the control experiments with normal inspired air

Further a cooling of the carotid blood was attempted (Fig 6) Although the temperature between the cooling capsules and the skin was held at about 6 C for 15 min in the steady state period of work there was only a small effect on the internal temperatures and on the rate of weight loss

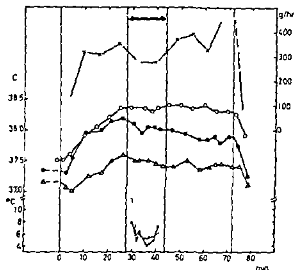


Fig 6 Cooling of the skin above the carotid arteries during the steady state of work at an intensity of 720 kpm per min Period of cooling shown by ←→ Temperature between skin and cooling capsules on both sides of the neck shown at the bottom of the figure
(×) rate of weight loss
(○) rectal temperature
(●) deep esophageal temperature
(△) tympani temperature
Subject H N

Discussion

The experiments here presented show that except at the very beginning of work the changes in sweat production during and after a work period is closely correlated in time to changes in internal temperatures expressed as the deep esophageal temperature and the tympanic temperature (Fig 1 and 2) At the same environmental temperature the different rates of sweating caused by different work intensities are linearly correlated to the observed changes in internal temperatures (Fig 3) So far the experiments are in agreement with the view that changes in the rate of sweating are regulated by the hypothalamic temperature (Benzinger 1959 and 1960) But the results of the experiments with work at constant rate at varying environmental temperatures (Fig 4) are in contrast to this theory This is possibly because the work intensity was much higher (900 kpm per min in our experiments versus 300 and 150 kpm per min in Benzinger's) and because part of our experiments were performed in cooler environments than Benzinger's In the present experiments (Fig 4) the rate of sweat secretion increased from 150 to 1 090 g per hr while the rectal temperature and the deep esophageal temperature which can be considered a good index of the aortic blood temperature remained constant At sweat rates between about 200 and 700 g per hr also the tympanic temperature seemed to remain unchanged Corresponding results were obtained in experiments with another subject The esophageal temperatures scatter considerably less than the eardrum temperatures and the determinations in Fig 4 together with a large material published earlier (B Nielsen and M Nielsen 1962) show with great probability that the steady state work level of aortic blood temperature — inside a wide range — is independent of the environmental temperature It might be thought that a cooling of the arterial blood takes place during its passage from the trunk to the head and that this cooling might be different under various conditions However experiments in which the subjects breathed air saturated with water vapor at body temperature showed that the elimination of the cooling from the respiratory passages did not increase either the rate of sweat secretion or the tympanic temperature (Table I)

Further a strong cooling of the skin above the carotid arteries (temperature 5—6 C between skin and cooling capsules for 15 min during the steady state of work) had only a small effect on sweat secretion and practically no effect on the tympanic temperature It seems therefore reasonable to assume that the variations found in sweat secretion at constant work rate are not caused by changes in hypothalamic or other internal temperatures That large increases in heat dissipating responses can occur without any increase in hypothalamic temperature are shown in recent experiments by Hammel *et al* (1963) who found that resting dogs panting in hot environments had the same or even a lower hypothalamic temperature than when shivering in the cold

While the internal temperatures and therefore probably the stimulus for the central receptors in hypothalamus at the same work intensity at varied environmental temperatures remains constant the skin temperature varies considerably and Fig 5 shows that the rate of sweat secretion increases nearly linearly with an increase in mean skin temperature The changes in the rate of sweating in this condition might therefore be thought to be influenced by the changes in skin temperature

Benzinger in recent publications (1962 and 1963) points out that although the changes in the rate of sweat secretion are normally caused by changes in hypothalamic temperature a certain inhibition of the heat dissipating responses can be elicited from skin receptors at skin temperatures below the threshold for cold reception (about 33 C in Benzinger s experiments)

In our experiments with constant work rate carried out at varied environmental temperature the internal thermal stimulus for sweat secretion in the steady state (whether acting in the hypothalamus or elsewhere in the core of the body) remained at a constant raised level in spite of the large variations in sweat rate 150 to 1 020 g per hr) The mean skin temperature was not measured in the work experiment at 37 5 C but in all the work experiments at temperatures between 5 and 30 C it varied between 23 and 32 C *i e* it was always lower than the value (35 C) at which sweating began in the resting condition (Fig 5) If therefore the changes in sweat secretion are governed only by the internal temperatures and by the temperatures of the skin receptors (as expressed by the mean skin temperature) the entire variation in sweat rate (Fig 5) during work performed at a constant rate therefore seems to be due to a varied degree of inhibition from the skin receptors But it then seems strange that such a variation in inhibition has the effect that the internal temperature at the same work rate increases to the same level at so different conditions for heat loss The temperature of the skin receptors must of course be somewhat higher than the temperature of the skin surface above the receptors and this temperature difference must be the higher the greater the work intensity Based on experiments at rest and during work in warm environments Kerslake (1955) forwarded the hypothesis that the rate of sweat production may be controlled solely by the temperature of the nerve endings situated at about the level of the superficial plexus in the dermis However in our experiments (Fig 5) which are performed in cool or moderately warm surroundings the difference between mean skin temperature in rest and during work is so large at a certain rate of sweat production that the regulation of the rate of sweat production hardly can be explained by this hypothesis

As mentioned above (p 000) a linear relation exists between sweat rate and internal temperatures when different rates of work are performed at the same environmental temperature in which case the skin temperature remains constant The increase in sweat secretion in these conditions might therefore be caused wholly or at least partly

by the increase in internal temperature However the possibility exists that other factors related to the intensity of work contribute to the increase in sweat rate as proposed by Robinson (1949 and 1962) and that such factors also are of importance for the adjustment of the constant internal temperature level found when work of the same intensity is performed at various environmental temperatures The fact that the rate of sweat secretion increases almost immediately at the start of work whereas the rise of internal temperatures lags behind for a couple of minutes (Meyer et al (1962) Beaumont and Bullard (1963) and Fig 1 and 2 in this paper) points to the existence of such factors Experiments in a following paper (B Nielsen and M Nielsen 1965) indicate however that in the steady state of work neuromuscular factors are only of minor importance for the magnitude of the rate of sweating

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Acta physiol scand 1965 64 323—331

From the Zoophysiological Laboratory A University of Copenhagen Denmark

Influence of Passive and Active Heating on the Temperature Regulation of Man

By

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Abstract

Nielsen B and M Nielsen *Influence of passive and active heating on the temperature regulation of man* Acta physiol. scand 1965 *64* 323—331 —The effect of passive heating through diathermia on thermoregulatory responses is compared to the effect of active heating by exercise on a Krogh bicycle ergometer in 2 human subjects The rate of heat production in the two conditions was of the same magnitude (about 5 times the basal heat production) — The rectal temperature increased with passive heating to about the same level as during active heating and appeared within limits to be independent of the environmental temperature At the same mean skin temperature conductance and sweat rate with passive and active heating were much higher than during normal rest But in the two conditions of heating the rates of skin blood flow were equal and the rates of sweating not much different when compared at the same mean skin temperature The results indicate therefore that the main stimulus for the increase of heat dissipation during the steady state of work caused by the work itself is the increased internal temperature and that the changes in heat dissipation during work caused by changes in the environmental temperature within wide limits are mainly due to changes in skin temperature

Thermoregulatory responses to warm environments or to muscular exercise are generally thought to be governed partly by changes in the temperature of the blood acting on the thermosensitive regions of the hypothalamus and partly by afferent impulses from cutaneous thermoreceptors stimulated by increases in the skin temperature (cf reviews by Bazett 1949 Robinson 1949 and Hardy 1961)

In 1959 and 1960 Benzinger suggested that changes in heat dissipation exclusively depend on the temperature in the anterior hypothalamus but later (Benzinger 1963) pointed out that a certain inhibition of sweating occurs when the skin temperature becomes lower than the threshold for cold reception B Nielsen and M Nielsen (1964) pointed out that if the rate of sweating depends only on the internal temperature and on the temperature of the skin receptors (represented by the mean skin temperature) then the entire variation in sweat rate during work of constant intensity at environmental temperatures between 5 C and 30 C must be regulated solely by the varying degrees of inhibition from the skin receptors But it seems difficult from such a theory to explain

the fact that the internal temperature at the same work intensity increases to the same level independent of wide variations in environmental temperature and the possibility that also other factors related to the intensity of work take part in the temperature regulation during muscular exercise must be considered Robinson (1949 1962 1963) Meyer *et al* (1962) and Beaumont and Bullard (1963) conclude from their experiments that neuromuscular reflexes may be involved

To examine this possibility we have compared the thermoregulatory responses to active heating i e muscular exercise with the responses to passive heating i e diathermia The work rate was adjusted so that the heat production per minute was equal to the heat production per minute during diathermia (electrically induced heat plus metabolic heat)

Methods

In the work experiments the rectal temperature was measured thermoelectrically in 4 depths as described earlier (B Nielsen and M Nielsen 1962) In the experiments with diathermia it was found that thermoelectric measurements could not be performed because the thermo couple itself was heated in the induction field and the rectal temperature was therefore measured by means of a clinical thermometer (This method was inconvenient in the work experiments) Skin temperatures averaged from 15 different places and surrounding wall temperatures (means of 6 measurements of walls ceiling and floor) were determined by a thermoelectric radiation receiver (cf B Nielsen and M Nielsen 1964) Changes in skin blood flow were measured as changes in the heat conductance of peripheral tissues i e the total heat loss per m of body surface per C difference between rectal and mean skin temperature (Burton 1934 Winslow Herrington and Gagge 1939 and Hardy 1937) The work was performed on a Krogh bicycle ergometer and the sweat secretion and the gas exchange determined as described in the above mentioned article

In the experiments with electrical heating the subject was lying on rubber bands fastened to a wooden frame The frame was suspended from the Krogh balance (Krogh and Trolle 1936) for determination of the weight loss

The artificial heating was administered by means of 2 short wave therapy apparatuses (Ultratherm 603 H and Ultratherm 525 E Siemens) One induction cable was placed over the chest and abdomen and the other under the rubber bands under the corresponding areas of the back both in a distance of about 2 cm from the skin Each of the cables was connected to one apparatus The subject was protected from heat radiation from the short wave apparatuses by screens covered by aluminium foil

The heat production P during the electrical heating i e the electrically induced heat + the metabolic heat was determined as

$$P = C + R + E - \Delta H \qquad \text{(equat 1)}$$

where P = total heat production in Kcal/hr
C = convection heat loss »
R = radiation » » » »
E = evaporation » » » »
ΔH = change in body heat content » »

For determination of the convection and radiation heat loss the factor $k_{(C+R)}$ was measured in special experiments

$$k_{(C+R)} = \frac{C + R}{T_S - T_A}$$

where T_S = mean skin temperature
T_A = mean of air temperature and wall temperature

The difference between wall and air temperature was in all experiments less than 1 C (C + R) was calculated from the metabolic rate evaporative heat loss and change in body heat content at an ambient temperature of 28 to 30 C at which the subjects were in nearly thermal equilibrium with the environment For the work experiments the factor was determined at different values of $T_S - T_A$ In the experiments (C + R) was then determined from this factor and the measured values of T_S and T_A

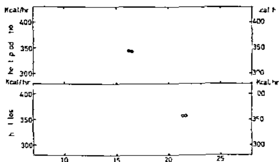


Fig 1 Rate of heat production and heat loss at different environmental temperatures Subject A J
● active heating
○ passive heating

The heat loss by evaporat on vas determined from the weight loss corrected for the we ght ch nge due to the resp ratory gas exchange using the value 582 cal/g for the heat of evaporatio The change in body heat content was calculated from the change in average body temperature multiplied by weight and by the specific heat of the body (0 83) The average body temperature T_B was calculated by means of *Burton s* formula $T_B = (0\ 35 \times T_{sk} + 0\ 65 \times T_{rectal})$

Procedure The subjects arrived in the morning semifasting rested fo 30 minutes at a comfortable temperature and then ente ed the climat c chamber wearing shorts only whereupon the electrical heating was started After a prelim nary period of heating of 30 min the d athermia was interrupted and measurements of we ght and rectal skin and wall temperatures we e performed as fast as poss ble (with n 5 m n) None of these measureme its we e possible wh le the short ave apparatuses were turned on The heat ng was then continued for the 60 m n experimental period and the measurements then repeated

The work experiments wer performed n a s milar manner with 30 min prelim nary work a d an experimental work period of 60 m n nterrupted by a pause of the same durat on as in the diathermia experiments During the paus and after the experimental periods measure m nts of we ght and rectal skin and wall temperatures were tak n t exactly the same t me intervals a in the diathe mia experiments During the 60 min experimental work period these measurements were epeated with regular ntervals

The heat product on in the work experiments was calculated in the same way as n the diathermia experim nts us ng the measurements taken in the pause and after the exper mental period but with the exception that the calculat on of the heat loss by rad at on + convect on was based on the sk n tempe atures m asu ed during work The reason for th is th t the sudd n cessation of mov ments changes the cond tions for the heat loss by convection and thus causes a rap d and cons derable increase of skin temperature In the diathermia experiments on the other hand the cl mati cond t ons does not change by the essation of the iduction of heat Control e perim nts n which skin temperatu es ere measured as f equently as poss ble during the first 10 minutes after nterruption of the l ctri al heating showed that the mean sk n temperature ncreased only about 0 1 C f om the interruption of the d athermia unt l the term na t on of the normal measurements This small hange could be disreg rded by the calculat on of the heat loss by con ection + rad at on

S n e th sweat secretion d m nishes rap dly after cessation of wo k and d atherm a the heat loss by ev porat on is sl ghtly und estimated because th weight loss determ ned n the pause nd afte the experiment l pe iod Due to the relatively long duration of the experimental period the error ho eve s only small and is the same in the work and n the diatherm a ex periments

As subjects were used ? oung male students (O A 21 years 77 kg 183 cm DuBo s area 1 98 m and A J 19 years 76 kg 185 cm DuBois a ea 1 99 m)

TABLE I

Subjects	Work intensity kpm/min	Heat production Kcal/hr		
		From O_2 uptake in work	From equation 1	
			Work	Diathermia
O A	700	403 8	372 4 n=8 S E = ±9 4	370 3 n=10 S E = ±6 4
A J	6[illegible]0	379 2	357 6 n=8 S E = ±5 7	357 2 n=12 S E = ±6 7

Results

Fig 1 shows the heat production from the experiments with electrical heating and from the work experiments in subject A J determined by means of equation 1 (see page 000) It is seen that the heat productions in the two kinds of experiments are equal at all temperatures There is a slight increase in heat production with increase of environmental temperature Since the heat loss both in subject A J (Fig 1) and in subject O A is practically independent of ambient temperature this small increase in heat production is possibly due to uncertainties in the determinations of the change in heat content The average values of heat production determined on basis of heat loss from the diathermia and the work experiments in both subjects are equal and in both subjects are about 7 % smaller than the heat production determined on basis of the oxygen consumption in the work experiments (Table I) This difference is mainly due to the fact that in the determination of the heat loss periods before and after the experimental

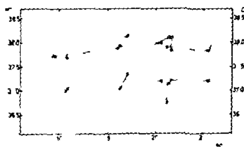


Fig 2 Rectal temperature in the steady state of the experiment at different environmental temperatures Subject O A
● active heating } Increased heat production
○ passive heating }
× rest before the experiment

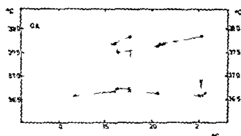


Fig 3 Rectal temperature in the steady state of the experiment at different environmental temperatures Subject A J
● active heating } Increased heat production
○ passive heating }
× rest before the experiment

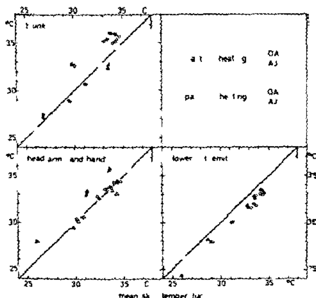


Fig 4 Regional skin temperatures (trunk — head arms and hands — lower extremities) plotted against mean skin temperature Two subjects A J and O A
Active heating solid symbols
Passive heating open symbols

period are included in which the heat loss is reduced because of the rapid decrease of sweat secretion after cessation of work and diathermia

When the heat production is the same the rectal temperature in the diathermia experiments increases to about the same level as in the work experiments and the raised temperature level appears to be independent of the environmental temperature inside the range of temperature studied (about 10 C to 24 C, Fig 2 and 3)

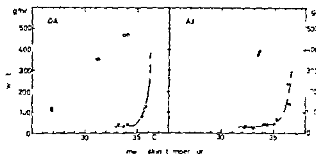


Fig 5 Sweat rate plotted against mean skin temperature.
● active heating } Increased heat production
○ passive heating
× stationary conditions of normal rest

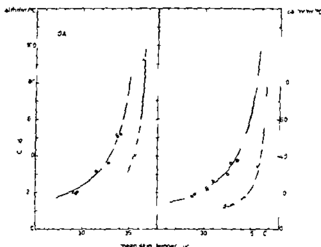


Fig 6 Conductance plotted against mean skin temperature
● active heating } Increased heat production
○ passive heating
× stationary conditions of normal rest

In Fig 4 the skin temperatures (average values of single measurements in both subjects) of 1) trunk 2) head arms and hands and 3) lower extremities are plotted against the corresponding mean skin temperatures from the experiments with active and with passive heating At the same mean skin temperature the skin temperature of the trunk is higher in the experiments with diathermia than in the work experiments and the average skin temperature of the head hands and arms are higher during work than during diathermia This also applies to the skin temperatures of the 3 regions head hands and arms separately The skin temperature of the lower extremities are somewhat lower than the corresponding mean skin temperatures and are at the same mean skin temperature almost the same in the work as in the diathermia experiments

However, the rectal temperature in the diathermia experiments was determined by a clinical thermometer and, therefore, possibly not quite as reliable as the thermoelectric measurements in the work experiments.

In Fig. 5 is shown the relationship between sweat rate and mean skin temperature in the normal rest experiments and in the experiments with active and passive heating. With increased heat production the sweat secretion is much higher than during rest, but at the same mean skin temperature there is only a relatively small difference in sweat rate in the diathermia and the work experiments. At the same mean skin temperature also the heat conductance of the peripheral tissues is much higher in the experiments with increased heat production than in the normal rest experiments. At the same mean skin temperature conductance is the same in the diathermia experiments and in the work experiments (Fig. 6).

Discussion

The present experiments have shown a remarkable similarity in thermoregulatory responses to passive heating by diathermia and to active heating by muscular exercise. At the same heat production (about 5 times the basal heat production) the rectal temperature reached about the same level in the diathermia experiments as in the work experiments and also, the increased temperature level was, within a wide range, independent of the environmental temperature. Further, at the same mean skin temperature, heat production, and rectal temperature, the skin blood flow (as measured by the heat conductance of peripheral tissues) was in the steady state increased to the same level in the two kinds of experiments whereas there was a small difference in sweat rate.

From a comparison of heat regulatory functions in rest and during exercise Robinson and co-workers (1962, 1963), Meyer *et al.* (1962) concluded that for all comparable values of, respectively, rectal temperature, mean skin temperature, mean body temperature, and $T_{Rectal} - T_{Skin}$, both sweat secretion and skin circulation were much higher during work than in rest and they suggest that neuromuscular reflexes take part in the temperature regulation in a manner similar to what is suggested for the regulation of respiration during work. From studies of sweating during passive exercise of a man's arms and legs, and from studies of the time relation between sweating and different internal temperatures, Robinson (1962) found it less likely that these reflexes were elicited from mechanoreceptors in the muscles and joints than from thermoreceptors in the working muscles, or in contact with veins draining the working muscles. Beaumont and Bullard (1963) studied local sweating on skin areas of forearm and calf by a method allowing continuous and very rapid recording of changes in rate of sweat secretion. They found that in warm environments ([illegible] °C), in which the subject was sweating in the pre-exercise period, the increase in sweat rate began after a delay of only 1.5 seconds after the start of work, and this rapid increase in sweat rate was correlated to the intensity of the work. The authors concluded that this rapid increase in sweating must depend on a neural stimulus, possibly an irradiation of descending motor impulses to centers involved in sweating. Such a mechanism has earlier been proposed by Krogh and Lindhard (1913) for the release of the initial changes taking place in respiration and circulation at the transition from rest to work.

The studies on the initial changes in sweat secretion and skin- and internal temperatures (Beaumont and Bullard 1963, Meyer *et al.* 1962, B. Nielsen and M. Nielsen 1965) point definitely towards the existence of a work factor of nervous origin, which causes

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Cardiovascular and Metabolic Response to Infusions of Prostaglandin E_1 and to Simultaneous Infusions of Noradrenaline and Prostaglandin E_1 in Man

Prostaglandin and related factors 35

By

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Abstract

Bergstrom S L A Carlson L G Ekelund and L Oro *Cardiovascular and metabolic response to infusions of prostaglandin E and to simultaneous infusions of noradrenaline and prostaglandin E_1 in man* Acta physiol scand 1965 64 332 339 — Prostaglandin E_1 (PGE) was infused at a rate of 0 1 -0 2 µg/kg/min into 3 healthy fasting male subjects for 30 min No consistent changes in arterial pressures occurred The heart rate increased about 20 beats/min The concentration of free fatty acids (FFA) and of glycerol in plasma increased A slight increase in oxygen consumption was observed PGE_1 infused simultaneously with noradrenaline to 2 healthy fasting male subjects reduced the increase in arterial pressures seen when only noradrenaline was infused to these subjects Furthermore PGE completely inhibited the noradrenaline induced bradycardia The increase in the concentration of FFA and glycerol in plasma caused by noradrenaline was only slightly reduced by PGE_1 PGE had no effect on the calorigenic effect of noradrenalin

We have recently observed that the prostaglandins F_1 E and E (PGF_1 etc inhibit the increase of blood pressure as well as the increase of the concentration of free fatty acids (FFA) and of glycerol in the plasma of anestethized dogs caused by catechol amines (Bergstrom Carlson and Oro 1964 a) It has also been shown that PGE_1 *in vitro* inhibits the stimulation of the release of glycerol from rat adipose tissue caused by catecholamines (Steinberg *et al* 1963 and 1964 a) Prostaglandins have been shown to cause a prolonged fall in blood pressure on i v injection in the rabbit and in the cat (Euler 1939) Bergstrom *et al* (1959) infused prostaglandin E to 2 human subjects and observed an increase in heart rate and a moderate fall in systemic arterial blood pressure and cardiac output

In this paper data will be reported on cardiovascular and metabolic effects of infusions of PGE$_1$ alone or together with noradrenaline in man. Preliminary reports have appeared on this work (Bergström *et al* 1964 b c)

Methods

Procedure

5 healthy male volounters age 23—33 years were studied. They reported at the laboratory at 8 a m after fasting over night. One catheter was placed into the brachial artery for sampling of blood and registration of pressures with an Elema differential transformer transducer (EMT 490 A) and recorded on a photokymograph (Klinik). The pressures were recorded over several respiratory cycles. The mean pressure was obtained by electrical integration (constant 0 ? sec)

Another catheter was placed into a brachial vein for the infusion. When ? i v infusions were given simultaneously one further catheter was placed into a vein in the other arm for this purpose. When PGE was infused i. a in subject 1 a second arterial catheter was placed in the aortic arch via the left brachial artery under X ray control. Due to an accident this catheter was withdrawn some centimeters and the tip of the catheter became situated in the distal part of the subclavian artery. All catheters were inserted percutaneously.

With all catheters in place the subjects rested comfortably in the supine position. No heparin was introduced into the blood stream. Blood samples were drawn into heparinized syringes, the blood centrifuged and the plasma immediately processed. Expired air was collected for periods of 6 min into Douglas bags. The gas volumes were estimated with a watertank spirometer. O_2 and CO_2 were estimated according to Haldane (193?)

PGE$_1$ was infused into one anesthetized dog during infusion of noradrenaline with the technique described previously (Bergström, Carlson and Orö 1964)

Material

The PGE$_1$ used was a crystalline preparation isolated from sheep glands as described earlier (Bergström and Sjövall 1960, Bergström *et al* 1962). PGE dissolved in ethanol was sterilized by ultrafiltration. This sterile solution of PGE$_1$ containing 50 / g ml was dispensed in 10 ml portions and stored at —15 C. Before infusion the solution was diluted about 10—?0 times with saline

Analytical methods

Plasma FFA was determined according to Dole (19?6) as modified by Trout *et al* (1960). Glycerol (Wieland 19?7) and glucose (Marks 19?9) were determined enzymatically. The plasma protein concentration was estimated with the Biuret method and showed no significant changes during the studies

Results

The results from the infusions of PGE$_1$ are given in Fig 1 and 2 and Table I and the result from the infusions of noradrenaline and PGE$_1$ in Fig 3 and 4 and Table II

Symptoms during infusion of PGE$_1$

During the i (infusion of PGE$_1$ *subject I* showed a slight reddening of the face. Immediately after the infusion slight intermittent abdominal cramps occurred. About 15 min after the infusion he complained of pulsating headache in the temples, difficulties to fixate the eyes and visual symptoms in the form of lightnings and other colored phenomenons. These symptoms continued for about 20 min. In this subject exactly the same symptoms were observed during the i a infusion of PGE$_1$. In addition the left arm showed intense flush and a pronounced edema developed within 10 min in the left hand. One hour after the infusion the left arm was slightly red and the hand still edematous

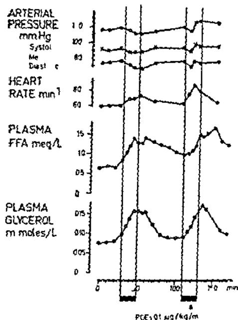


Fig 1 Effect of one i v and one i a infusion of PGE to subject 1 on arterial pressure heart rate concentration of FFA and of glycerol in arterial plasma

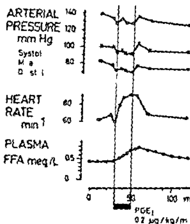


Fig 2 Effect of i v infusion of PGE to subject 3 on arterial pressure heart rate and the concentration of FFA in arterial plasma

Subject 2 did not have any objective or subjective symptoms during or after the infusion

Subject 3 started to flush in the face after 7 min of the infusion and complained of slight headache when PGE$_1$ had been infused for 15 min

Cardiovascular response to PGE$_1$

The systolic diastolic and mean arterial pressure remained almost unchanged during the infusions of PGE$_1$ The heart rate on the other hand increased about 20 beats per min in all subjects

TABLE I Oxygen uptake total ventilation and RQ before during and after i v infusion of PGE as descr bed in the text

Time minutes	40 before	10—20 before	4—8 after start	8—14 after start	60 after end
Subject 1					
Oxygen uptake ml/min	286	307	368	333	
Total vent lation l/min	6 87	8 20	10 55	7 53	
RQ	0 73	0 78	0 82	0 65	
Subject 3					
Oxygen uptake ml/min		296	319		306
Total ventilat on l/min		9 14	11 89		9 11
RQ		0 79	0 89		0 76

In relat on to infusion of PGE

Metabolic response to PGE_1

The concentration of FFA in plasma increased in all 3 subjects during the infusion of PGE_1 and continued to increase slightly for 10—20 min after the infusion in order then to level off slowly The average increase was 0 5 meq/l The concentration of plasma glycerol followed the same pattern initially but decreased more rapidly after the infusion (Fig 1)

The concentration of glucose in plasma increased from 90 to 105 mg per 100 ml during th 2 infusions in subject 1 but showed no significant changes in the other 2 per sons

The oxygen uptake increased about 20 per cent in subject 1 and 10 per cent in subject 3 during the infusion of PGE_1 (Table I) The RQ rose during the first 10 min of the infusion in these 2 subjects simultaneously with an increase in the total ventilation In subject 1 the RQ fell from 0 82 to 0 65 during the last 10 min of the infusion at which time the total ventilation decreased to the preinfusion values

Symptoms during infusion of noradrenaline and PGE_1

Both subjects were pale during the infusion of noradrenaline and complained of opres sion and a feeling of tightness in the chest During the simultaneous infusion of PGE_1 and noradrenaline the noradrenaline induced symptoms were abolished and the subjects showed a very slight reddening of the face *Subject 5* furthermore complained of slight abdominal cramps

Card ovascula response to norad enaline and PGE_1

Th increase in systolic diastolic and mean arterial pressure elicited by the first infusion of noradrenaline was reduced by approximately 50 per cent when noradrenaline and PGE_1 were infused together The bradycardia usually seen during infusions of nor adrenaline was completely inhibited by PGE_1 and there was a tendency to increase in heart rate when the two agents were infused together

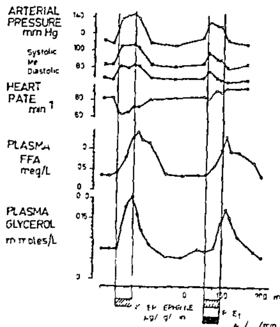


Fig 3 Effect of one i v infus on of noradrenaline and one i v infus on of both noradrenal ne and PGE_1 to subject 4 on arterial pressure heart rate concentra tion of FFA and of glycerol in arterial plasma

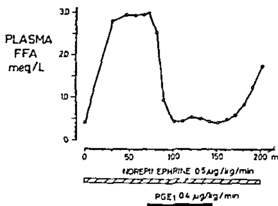


Fig 4 Effect of PGE on the nor adrenal ne induced increase in concentration of FFA in arterial plasma in one anesthetized dog

Metabolic response to noradrenaline and PGE_1

The rise in plasma FFA induced by noradrenaline was slightly less when also PGE_1 was infused into the 2 subjects In subject 4 the FFA level increased to 1 4 meq/l with noradrenaline and 1 3 m q/l with noradrenaline and PGE_1 The corresponding data in subject 5 were 1 2 and 1 05 meq/l The same trend was found for the increase in plasma glycerol However infusion of PGE_1 of the same batch to a dog during contin uous infusion of noradrenaline and with a similar ratio noradrenaline/PGE_1 caused a striking fall in the elevated plasma FFA levels (Fig 4)

TABLE II Oxygen uptake total ventilation and RQ before and during infusion of noradrenaline (infusion A) and simultaneous infusion of noradrenaline and PGE_1 (infusion B) as described in the text

Time minutes	15 before A	5—10 before A	8 after start A	14 after start A	60 before B	40 before B	8 after start B	14 after start B
Subject 4								
Oxygen uptake ml/min	281	256	296	310	240	257	307	310
Total ventilation l/min	7 49	7 76	9 03	9 05	7 26	7 59	8 99	8 42
RQ	0 79	0 76	0 77	0 75	0 78	0 78	0 79	0 73
Subject 5								
Oxygen uptake ml/min	266	273	312	324	272	277	327	339
Total ventilation l/min	7 95	8 43	10 15	9 90	8 34	7 97	10 60	9 60
RQ	0 78	0 80	0 82	0 80	0 77	0 80	0 81	0 76

In relation to the infusion

The plasma glucose concentration increased during the infusion of noradrenaline from 80 to 102 and from 75 to 103 mg per 100 ml respectively in the 2 subjects During the simultaneous infusion of noradrenaline and PGE_1 the corresponding figures were from 79 to 94 and from 70 to 87 mg per 100 ml

The oxygen consumption increased 15—20 per cent during the infusions of noradrenaline (Table II) The same increase in oxygen consumption was observed when noradrenaline and PGE_1 were infused together (Table II)

Discussion

Infusion of PGE_1

The flush of the face and the headache we observed during infusion of PGE_1 confirms earlier findings in man (Bergstrom *et al* 1959) The increase in pulse rate was of similar magnitude as found by Bergstrom *et al* (1959) In the present study however we found no decrease in the arterial pressures during infusion of PGE_1 contrary to the earlier study in man This discrepancy might be due to the greater amounts of PGE infused by Bergström *et al* in 1959 The unchanged arterial pressures and the increased heart rate suggests either that the stroke volume decreased or that a hyperkinetic circulation appeared as a compensation for a vasodilation In this connection it is of interest to note that prostaglandin had no effect on the heart lung preparation of the cat while a vasodilator effect was demonstrated in the perfused hind leg of the cat (Euler 1939)

However Bergström *et al* (1959) found a decrease of the stroke volume in their studies in man

The increase in the concentration of FFA in plasma was surprising since it has been shown that PGE_1 *in vivo* inhibits the catecholamine induced increase in FFA in anesthetized dogs (Bergstrom *et al* 1964 a Steinberg *et al* 1964 a) and also reduces the basal level of FFA in non anesthetized dogs (Steinberg personal communication) Furthermore PGE_1 inhibits the basal as well as the catecholamine stimulated release of glycerol from rat adipose tissue *in vitro* (Steinberg *et al* 1963 1964 a) This different response to PGE_1 in man and in dogs and rats may be due to a species difference with regard to a direct effect of PGE_1 on the FFA metabolism or it may be due to different secondary effects of PGE_1 in the species Finally differences in experimental design may be of importance for the discrepancy in results in these studies

Simultaneously with the increase in the concentration of FFA the concentration of glycerol in plasma increased This suggests that the mobilisation of FFA from adipose tissue was enhanced when PGE_1 was infused due to stimulation of the lipolysis of the triglycerides stored in adipose tissue

The increase in oxygen consumption observed during infusion of PGE_1 can to some extent be ascribed to the increased work necessary for the increased ventilation Part of the increased oxygen consumption may be due to the increased concentration of FFA in plasma as has been discussed in connection with the calorigenic action of catecholamines (Havel *et al* 1964 Steinberg *et al* 1964 b)

Infusion of PGE_1 and noradrenaline

In previous studies from these laboratories we have utilized a similar technique with two infusions of noradrenaline wich were 3 hours apart (Havel *et al* 1964) In these studies the response to the second infusion of noradrenaline was quantitatively as well as qualitatively identical to the response of the first infusion with regard to arterial pressures as well as to increase in plasma FFA and increase in plasma glucose These findings justify the use of this technique for quantitative as well as qualitative evaluations on the effect of various agents on noradrenaline induced changes

The present study showed that the pressor effect of noradrenaline was inhibited to about 50 per cent with PGE_1 and that the bradycardia was abolished The inhibition of the bradycardia may partly be ascribed to the reduced pressure response and partly due to a direct stimulation of the heart rate by PGE_1

The effect of PGE_1 on the response of plasma FFA and glycerol to noradrenaline was much less pronounced than previously found in dogs (Bergstrom *et al* 1964 a) This is also apparent from Fig 4 which shows that in an anesthetized dog PGE_1 completely inhibited the effect of noradrenaline on plasma FFA If the reason for this difference is due to the species differences or due to the different experimental techniques needs further studies to be evaluated

PGE_1 had no effect on the calorigenic effect of noradrenaline This is in conformity with earlier studies suggesting that the calorigenic action of noradrenaline in man is at least partly related to the stimulation of mobilization of FFA from adipose tissue (Havel *et al* 1964 Steinberg *et al* 1964 b)

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Acta physiol scand 1965 64 340—344

From the Department of Pharmacology University of Lund Sweden

Potassium Induced Release of Transmitter at the Human Neuromuscular Junction

By

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Abstract

Elmqvist D *Potassium induced release of transmitter at the human neuromuscular junction* Acta physiol scand 1965 64 340—344 — When the release of ACh quanta from the human intercostal motor nerve terminals recorded as miniature end plate potentials (m e p p s) was enhanced by raising the potassium ion concentration the increased rates were only sustained if release rates were limited to not more than 150 m e p p.s/sec either by not using higher concentrations of KCl than 30 mM or by reducing release with increased magnesium and lowered calcium concentrations when higher potassium ion concentrations were used If higher rates of release were allowed to occur in the presence of 40 or 50 mM KCl the release rates subsequently declined to very low levels The total number of quanta released by 40 or 50 mM KCl corresponded to the amount of ACh estimated to be present in the motor nerve terminals

It is well known that raising the external potassium ion concentration greatly increases the frequency of miniature end plate potentials (m e p p s) at the neuromuscular junction (Liley 1956) As the m e p p s are elicited by quantal liberation of acetylcholine (ACh) from motor nerve terminals an increase in m e p p frequency reflects an increased rate of release of ACh

During prolonged tetanic stimulation of the motor nerve the end plate potentials progressively decline in amplitude (del Castillo and Katz 1954 Brooks and Thies 1962) That this effect is due to a decrease in the number of quanta in the end plate potentials has recently been shown (Elmqvist and Quastel 1965b) It would therefore be of interest to find out whether or not the motor nerve endings would be capable of sustaining high rates of transmitter release during intense stimulation with potassium ions It will be shown that human nerve endings are only capable of sustaining increased rates of release induced by external potassium ion concentrations up to 30 mM If the potassium ion concentration is increased still further a higher initial rate is followed by a decline in the m e p p frequency to very low levels

Methods

The experiments were performed on human intercostal muscle obtained at thoracotomy from patients with no known muscular or neuromuscular disease The specimens were dissected and mounted as previously described (Elmqvist *et al* 1964)

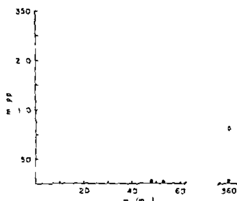


Fig 1 The average m e p p frequency at 5 min intervals at single human intercostal neuromuscular junctions plotted against the time after enhanced release was started □ KCl 30 mM ● KCl 40 mM ○ after 1 hr in Ca free solution with EDTA 1 mM and KCl 5 mM and then 2 5 hrs in Ca free solut on with EDTA 1 mM and KCl 50 mM at 0 time release of ACh quanta was started by removing EDTA and adding 2 mM $CaCl_2$, △ 50 mM KCl in a preparation in wh ch release was l mited with 8 mM $MgCl_2$ and 1 mM $CaCl_2$ The average m e p p frequency and 2 × S D in 10 fibres after 6 hrs in 30 mM KCl and 40 mM KCl are also shown

The usual techniques for intracellular recording with KCl filled glass cap llary microelectrodes were employed (Fatt and Katz 1951) The microelectrodes used were 3—8 MΩ in resistance when measured in 3 M KCl Potentials were observed on the oscilloscope screen and recorded on paper using an Elema Mingograf 81 A DC reco d at low amplification and AC records (time constant 1 sec) at three higher amplifications were registered s multaneously

Th no mal bathing fluid had the following co nposition (mM) NaCl 135 $NaHCO_3$ 15 $NaHPO_4$ 1 0 KCl 5 0 $CaCl_2$ 2 0 $MgCl_2$ 1 0 glucose 11 0 and was bubbled with 95 O and 5 CO with a resulting pH of 7 2 –7 4

Changes in KCl concentration were accompanied by approp iate reduction n NaCl concentrat on to keep the solution iso-osmolar except in earlier experiments in this series in w h ch no alteration in NaCl oncentration was made when the KCl concentration was increased there was no difference in the esults whether or not the sodium concentrat on was modified

To avo d changes in quantum size consequent upon the intense stimulation of transmitter release used chol ne chloride 5 × 10 g/ml was always added to the bathing solution (Elmq ist and Quastel 1965a) Experiments were carried out at a temperature of 32 C

M e p p frequency was determined by whenever poss ble counting at least 100 m e p p s when the frequency was higher than 100/sec the m e p p s occuring in 1 sec w ere counted.

Results

As has been previously reported (Elmqvist Johns and Thesleff 1960) the resting frequency of m e p p s in human intercostal muscle was about 0 2 sec If the potassium ion concentration was raised abo e 10 mM the frequency of the m e p p s greatly increased

By measuring the mean m e p p frequency in about 10 fibres at different potassium ion concentrations it was found that in human intercostal muscle as in the rat diaphragm (Liley 1956) the logarithm of the m e p p frequency was linearly related to the logarithm of the potassium ion concentration as is also the calculated depolarization of the motor nerve terminals (Liley 1956 Katz 1962)

That the m e p p frequency produced by 30 mM potassium was well sustained was shown by experiments in which mean m e p p frequency was determined 30 min after the potassium concentration was increased and then again afte about 5 hrs In both cases the m e p p frequency was about 150 sec In Fig 1 are shown the time course of the change in m e p p frequency produced by 30 mM KCl at a single junction (□) and the average m e p p frequency in ten fibres after 6 hrs in the raised potassium ion concentration

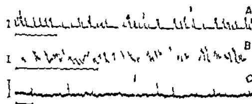


Fig 2 Intracellular record of m e p p s at a single human intercostal neuromuscular junction 2 min (A) 6 min (B) and 5? min (C) after the potassium concentration was raised from ɔ to 40 mM R P was in A 5? mV B 4? mV and C 3? mV Calibrations Voltage 1 mV time 200 msec

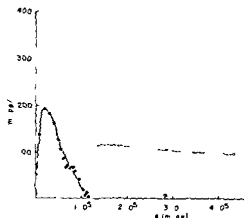


Fig 3 M e p p frequency at single human intercostal neuromuscular junctions plotted against the total number of m e p p s recorded after the initiation of rapid release Symbols are the same as in Fig 1 The junction at which release was stimulated with 50 mM KCl was the same as is depicted in Fig 1 The first 15 points and in the experiment with 30 mM KCl the first 10 represent data obtained at 1 min intervals subsequent points represent means over 5 min periods

If the potassium concentration was increased to 40 (●) or 50 (○) mM the initial rise in m e p p frequency was greater than that produced by 30 mM However this high frequency was only present for a short period of time and the rates subsequently declined to very low levels often approaching the resting frequency The decline in m e p p frequency was real and not secondary to the m e p p s getting smaller with consequent loss in the base line noise (Fig 2) The decrease in m e p p amplitude seen in some fibres was fully explained by the depolarizations of the muscle fibre membrane produced by the high potassium ion concentration and the damage to the cells produced by the microelectrode insertions (Katz and Thesleff 1957)

As the nerve terminals were able to release quanta at a moderate rate for very long periods of time it is unlikely that the nerve terminal was incapable to make new ACh quanta in the bath and could only release those already present when the muscle was removed from the patient In the very high potassium ion concentration this number of quanta should then be released in a much shorter period of time than with lower potassium concentrations That this was not the case is clearly shown in Fig 3 where m e p p frequency produced by 30 40 and 50 mM KCl at 3 different junctions has been plotted against the total number of quanta released after stimulation was initiated The figure demonstrates that in the presence of 30 mM KCl the nerve terminal had released more quanta than it did with 40 and 50 mM KCl in the 75 min period over which the release from these fibres was followed

The difference becomes much more pronounced when the release over longer periods are estimated For instance after six hours in 30 mM KCl the average frequency of m e p p s was still the same as after a few minutes in that solution Therefore about 2 5 million quanta had been released during the 6 hr period In the preparations treated

with 40 mM KCl on the other hand after an initial high frequency discharge which released about 200 000 quanta the continued release at about 4/sec only contributed an additional 100 000 quanta so that the total quantal output in a 6 hr period was only about one tenth of the amount released by 30 mM KCl in the same period of time

The total number of m e p p s recorded after the application of 40 or 50 mM KCl varied considerably from fibre to fibre but no systematic difference between the two concentrations was detected The mean of 4 fibres in 40 mM KCl was 216 000 $\pm$ 67 000 ($\pm$ S D) and the mean of 5 fibres in 50 mM KCl was 225 000 $\pm$ 126 000

In human intercostal muscle as in the rat diaphragm (Hubbard 1961) raising the magnesium and lowering the calcium ion concentration reduced the increase in m e p p frequency produced by raised potassium ion concentrations and if calcium was completely removed the enhancement in m e p p frequency was completely abolished

By utilizing this fact it was possible to expose muscle to high potassium ion concentrations for several hours without a simultaneous release of quanta at an increased rate Preparations of human intercostal muscle were washed in a calcium free bathing solution with 1 mM ethylene diamine tetraacetic acid (EDTA) (normal potassium ion concentration) After 1 hr the specimens were transferred to solutions containing increased potassium ion concentrations still without calcium and with EDTA After an additional 1 to 3 hrs transmitter release was started by removing EDTA and adding 2 mM $CaCl_2$ The rates of release and the time course of rate changes were similar to those seen when transmitter release was increased by simply increasing the potassium ion concentration with calcium present all the time The total amounts of transmitter released were also the same

As extended exposure to the high potassium ion concentrations did not prevent high frequency release the possibilities remain that these potassium ion concentrations in themselves blocked the formation of ACh quanta at an early stage or that the high release rates attained in them were responsible for the subsequent decline in m e p p frequency

The simple presence of 40 or 50 mM potassium does not limit the amounts of ACh that can be released This is shown by experiments in which the m e p p frequency produced by 50 mM KCl was limited to about 1?0 m e p p s sec by increasing the Mg concentration to 8 mM and reducing the Ca concentration to 1 mM In these experiments the release rates did not decline with time (Fig 1)

When the release rates in 40 or 50 mM KCl had declined to about the resting level treatments known to increase m e p p frequency such as further addition of potassium elevation of the calcium concentration or inducing hyperosmolarity of the bathing solution were undertaken These manipulations only had a very small and brief effect on the m e p p frequency so that at most a few thousand additional m e p p s were elicited

Discussion

The phenomenon of decreasing rates of release of ACh quanta in the presence of high potassium ion concentrations only occurred when very high release rates were produced by 40 or 50 mM KCl It did not occur when release produced by these concentrations of potassium was prevented or reduced by alterations in the calcium magnesium levels Thus it could not be the prolonged exposure to very high potassium ion concentrations alone which limited the release of ACh quanta

If the release rates were limited to an upper limit of about 150 m e p p s/sec either by causing release with up to 30 mM KCl or by the addition of magnesium or removal of calcium when higher potassium concentrations were used the nerve terminals were capable of releasing at least 2 5 million quanta After the release of this great number of quanta the release rates were not reduced and it is therefore likely that they would have been capable of keeping up release at this moderate rate indefinitely As the total amount of releasable ACh in the motor nerve terminals only amounts to the equivalent of about 200 000 quanta (Elmqvist and Quastel 1965a) it must be concluded that the nerve endings were able to make new ACh quanta in the bath

The number of ACh quanta which could be released from the motor nerve terminals with 40 and 50 mM KCl and normal calcium magnesium levels varied considerably from fibre to fibre with an average of 221 000 ± 99 000 (mean ± S D 9 fibres) This figure is similar to the total amounts of releasable ACh present in the motor nerve terminals determined by using hemicholinium no —3 to block the synthesis of ACh (Elmqvist and Quastel 1965a)

This correlation between the amounts of ACh that can be released after ACh synthesis has been blocked by HC-3 and that which can be released by 40 and 50 mM KCl may be largely fortuitous but the possibility that the very high release rates produced by 40 or 50 mM KCl may in some way have interfered with the formation or release of newly formed ACh quanta must be considered

The mechanism of such a block is however obscure That the rate of quantal release was affected rather than the quantum size indicated that it was not a block of ACh synthesis similar to that produced by HC-3 but that it could occur in the process of quantal formation or in some later step prior to release or even at the release site

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Inhibitory Action from the Flexor Reflex Afferents on Transmission to Ia Afferents

By

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Abstract

Lund, S., A. Lundberg and L. Vyklický. *Inhibitory action from the flexor reflex afferents on transmission to Ia afferents.* Acta physiol. scand. 1965. 64. 345—355. — In spinal cats the dorsal root potential (DRP) evoked from Ia afferents of flexors can be effectively depressed by volleys in the flexor reflex afferents (FRA). It has been confirmed that in the acute spinal cat volleys in Ia afferents of flexors give a primary afferent depolarization (PAD) in Ia afferents but not in Ib and cutaneous afferents and that volleys in the FRA do not depolarize Ia afferents. Excitability measurements and intracellular recording from Ia afferents have shown that the PAD evoked from Ia afferents is depressed from the FRA. The presynaptic inhibition from Ia afferents of the Ia EPSP in motoneurones can be removed by volleys in the FRA. It is concluded that volleys in the FRA inhibit transmission from Ia to Ia at an interneuronal level possibly presynaptically through depolarization of interneuronal terminals. The findings are discussed in relation to the function of the gamma loop in the flexor reflex.

Modern reflex physiology has been concerned with the analysis of reflex actions from different systems of primary afferents and little attention has been given to the problem of reflex interaction. In classical reflex physiology this was one of the main problems and a well known example of inhibitory interaction is the exclusion of the scratch reflex during the flexor reflex (Sherrington 1906). Sherrington (1906) considered "the compounding of reflexes" to be an essential part in integration.

Interaction between the central actions of different afferent systems can be provided by such examples of primary afferent depolarization (PAD) in which the giving and receiving primary afferents are different, so that the presynaptic inhibition does not constitute a simple negative feed back (cf. Eccles 1964). However, interaction at the primary afferent level presumably affects transmission to a number of central pathways from a given primary afferent system. Selective action on a specific pathway can probably only be exerted through inhibition of the interneurones of this pathway. An example of reflex interaction of the latter type will be given in the present paper. A preliminary report of some of the results has appeared (Lundberg and Vyklický 1963).

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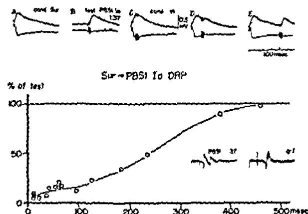


Fig. 1. The upper traces in A–F are dorsal root potentials (DRP) recorded from the most caudal dorsal rootlet in L6. The lower traces are recorded from the L7 dorsal root entry zone. In A there is stimulation of the sural nerve and B shows the test DRP evoked from a train of maximal Ia volleys from PBSt. The separation in the Ia and Ib volleys is shown in the inset records of the graph with double volleys being employed in the left record and the first stimulus just maximal for Ia afferents. The stimulus strength is indicated in multiples of threshold for the nerve. In C–E there is combined stimulation of the sural and the PBSt nerve. The depression of the test Ia DRP is plotted in the graph. Chloralose anaesthesia.

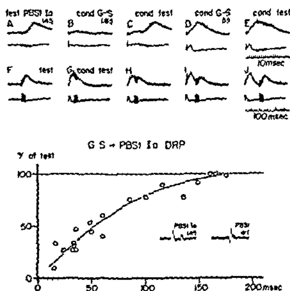


Fig. 2. As in Fig. 1 but with conditioning of muscle afferents. A shows the testing Ia DRP from PBSt. The stimulus strength given in multiples of threshold for the nerve is submaximal for Ia. The inset records in the graph show the separation in the Ia and Ib volleys. Double volleys are given in the left record and the first stimulus at 1.45 × threshold is maximal for Ia but subthreshold for Ib afferents. The DRPs in B and D are obtained on stimulation of the G-S nerve at group I strength and at group III strength respectively. In C there is no depression of the test Ia DRP by a group I volley in G-S. In I–J is shown the depression from high threshold muscle afferents. The fast speed is used in A–E and the slow speed in F–J. Chloralose anaesthesia.

Methods

The experiments were made on spinal cats anaesthetized with chloralose or unanesthetized decorticated spinal cats (cf. Voorhoeve 1960). For recording of dorsal root potentials, excitability measurements and intracellular recording from axons and motoneurones, cf. earlier papers (Lundberg and Voorhoeve 1962, Carpenter et al. 1963, Carpenter, Lundberg and Norrsell 1963). All experiments were made on cats in which the proximal nerves of the limb displayed separation in Ia and Ib volleys (Eccles, Eccles and Lundberg 1957).

The following abbreviations are used: quadriceps Q, posterior biceps-semitendinosus PBSt, anterior biceps-semimembranosus ABSm, gastrocnemius-soleus G-S, flexor digitorum longus FDL, tibialis anterior, extensor digitorum longus (deep peroneal) DP, sural Sur, superficial peroneal SP, dorsal root potential DRP, primary afferent depolarization PAD.

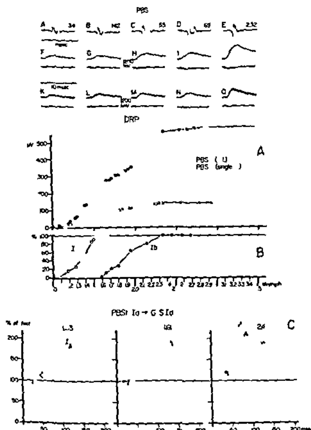


Fig 3 The upper traces n F—O are DRPs ecorded from the most caudal dorsal rootlet n L6 The lower traces in F—O and the traces in A—E are recorded from the L7 dorsal root entry zone The double volley technique is employed in A—D to show the separation n the Ia and Ib volleys The PBSt nerve is st mulated and the strength is given n multiples of the threshold above each column of records In the upper graph (A) the he ght of the DRP is plotted in µV as a funct on of the stimulus strength as sho n n F—J f r a tra n of olleys and in K—O for single volleys Graph B with the same absci sa s show s the si e of the Ia ( ) and Ib (●) incom ng volley plotted against sti nulus strength Observe that with s ngle volley most of the DRP is from Ia afferents and with a tra n of volleys more than half is evoked from Ia afferents The lower th ee graphs (C) a e f om the same experiment and show intraspinal excitab l ty measurements from Ia afferents of G S A n croelectrode was nse ted into the motor nucleus of G S and the test sp ke recorded peripherally in the G S nerve The increase n e c tab l ty was obtained with a short train of volleys in the PBSt nerve at the st ength indi ated abov each gr ph Unanaesthetized decorticate sp nal cat

Results

D f e ion of the Ia DRP

The DRP in B Fig 1 is evoked by a train of maximal Ia volleys from PBSt the separation in Ia and Ib volleys being shown in the inset records in the curves of Fig I Following the conditioning volley in the sural nerve (that alone evokes the DRP in A) there is a very effective depression of the Ia DRP shown at different intervals in C—F

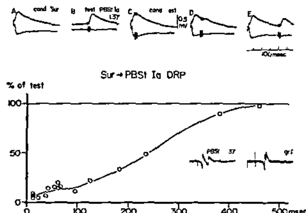


Fig 1 The upper traces in A—E are dorsal root potentials (DRP) recorded from the most caudal dorsal rootlet in L6 The lower traces are record d from the L7 dorsal root entry zone In A there is stimulation of the su ral nerve and B shows the test DRP evoked from a train of max imal Ia volleys from PBSt The separation in the Ia and Ib vol leys is shown in the inset records of the graph with double volleys being employed in the left record and the first stimulus just maxi mal for Ia afferents The stimu lus strength is indicated in mul tiples of threshold for the nerve In C—E there is combined stimulation of the sural and the PBSt nerve The depression of the test Ia DRP is plotted in the graph Chloralose anaesthesia

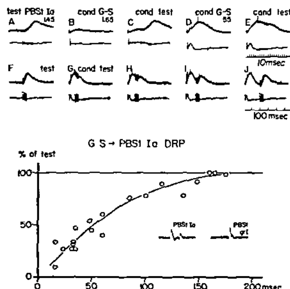


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The following abbreviat ons are used quadriceps Q posterior biceps-semitendinosus PBSt semitendinosus ST anterior biceps-sem membranosus ABSm gastrocnemius-soleus G S flexor d gitorum longus FDL tib alis anterior extensor d gitc rum longus (deep pero neal) DP sural Sur superficial peroneal SP dorsal root potent al DRP primary afferent depolarization PAD

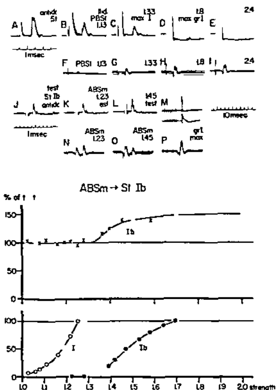


Fig 5 The test of intraspinal excitability in Ib fibres was performed through a microelectrode inserted into the intermediate nucleus where the Ia and Ib focal potentials were maximal A shows the antidromic response recorded peripherally in the semitendinosus (St) nerve It has three components and in B—E a colliding volley shown in the corresponding lower records F—E was evoked from another pair of electrodes on the PBSt nerve The first component decreases in B and has disappeared in C with the maximal Ia colliding volley The second component disappears when the colliding volley in addition includes Ib afferents and the third component disappears when the strength of stimulation is raised above group I maximum so that the low threshold group II afferents are included in the colliding volley In J—M there is excitability measurement in Ib afferents A maximum Ia volley was used for collision so that the remaining antidromic response is in Ib and group II afferents A train of maximal Ia volleys from ABSm has no effect in K but there is an increased Ib excitability in L when also Ib afferents from ABSm are stimulated M shows the time relationship between the conditioning and testing stimuli The separation in Ia and Ib volleys from ABSm is shown in M—P The upper curve shows the excitability as a function of stimulus strength and the lower graph shows the size of the Ia and Ib volleys as a function of stimulus strength The abscissa for the two graphs is the same The records in A—P consist of superimposed traces Unanaesthetized decorticate spinal cat

records A—E illustrating the separation in Ia and Ib volleys F—J the DRPs evoked by a train of volleys K—O the DRPs evoked by single volleys All records were evoked at the strengths indicated above each column in multiples of the threshold 1 55 being maximal for Ia and subthreshold for Ib The relationship between the size of the DRP to the incoming Ia and Ib volleys is shown in curve A Of the DRP evoked by a train of group I volleys more than half is caused by impulses in group Ia afferents (F—J and ● in graph A) and with single volleys Ia afferents contribute an even larger fraction (K—L and × in graph A) Excitability measurements from Ia afferents at the different strengths indicated in the lower curves show that in this case most of the primary afferent depolarization in Ia afferents was evoked from Ia afferents There was no difference in receptiveness between Ia afferents from flexors and extensors

Fig 4 illustrates that the depression of the Ia EPSP in motoneurones may be evoked mainly by Ia afferents The intracellular unconditioned homonymous Ia EPSP in a G-S motoneurone is shown in E The depression in G is caused by volleys in Ia affer

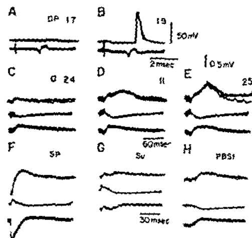


Fig 6 The upper traces are intracellular (KCl electrode) from a DP axon The low amplification is used in A and B the high in all other records The lower traces in C—H were obtained after withdrawal of the electrode to a just extracellular position In each record the extracellular potential should be subtracted from the intracellular in order to measure the PAD Lower traces in A and B and middle traces in C—H are from the L7 dorsal root entry zone In record A the stimulus strength was maximal for group I (tested with the double volley technique) The axon had its threshold at 1 85 × threshold and B is taken at 1 9 × threshold Hence the axon is a group II afferent There is no PAD evoked from group I afferents of Q and PBSt (C and H) but high threshold afferents from Q give a clear action (D and E) and there is also an effect from the cutaneous nerves (F and G) All records consist of superimposed traces Chloralose anaesthesia

ents from PBSt and the additional effect in H by volleys in Ib afferents from this nerve The actions from Ia afferents of flexors are larger than described by Eccles Eccles and Magni 1961 and Eccles Magni and Willis 1962 and this may be related to the fact that unanaesthetized preparations or cats under chloralose were used in the present experiments There can however be no doubt that also Ib afferents from flexors contribute to the PAD in Ia afferents this is apparent in Fig 3 and 4 but in some experiments the effect from Ib afferents was larger than from Ia as was usually found by Eccles *et al* (1962

Excitability measurements were also made from cutaneous afferents and from Ib afferents Confirming Eccles *et al* (1963 b c) no action was found from Ia afferents of flexor and extensor nerves to neither of these afferent systems as is illustrated in Fig 5 for the effect from group Ia and Ib afferents from ABSm on Ib afferents from semitendinosus Intracellular recording has been made from four group II afferents and neither of them received any PAD from Ia or Ib afferents (Fig 6)

Under special conditions (after Dopa) volleys in the FRA can give PAD in Ia afferents (Andén Jukes Lundberg and Vyklicky 1964) but we have never found such an action in the normal acute spinal cat (Fig 9) There was regularly an action from the FRA on cutaneous afferents (cf Eccles *et al* 1963 c Carpenter Engberg *et al* 1963) and in the group II muscle afferents a similar action from the FRA was met with (Fig 6)

It has not yet been proved that the Ia afferents from flexors evoke PAD exclusively in Ia afferents but the fact that the Ia DRP can be almost completely depressed from the FRA suggests that there is an inhibition of the pathway to Ia afferents

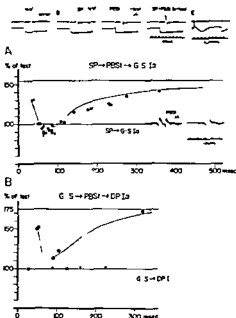
Depression of the PAD in Ia afferents

Excitability measurements The excitability of Ia afferents from flexors and extensors was investigated through stimulation of them by a microelectrode inserted into the motor nucleus of G S or PBSt and recording in the periphery from the corresponding nerves Records and curves from such experiments are shown in Fig 7 The test response recorded in the G-S nerve is shown in record A and the facilitation from PBSt Ia afferents

Fig 7 Intraspinal excitability measurement from Ia afferents from G S A microelectrode was inserted into the G S motor nucleus The test response (A and 100 in graph A) was recorded in the G–S nerve B and filled circles (●) in graph A show the effect of conditioning volley in the SP nerve The facilitation in C and 153 on the ordinate of the graph (A) is evoked by a train of PBSt Ia volleys Inset records in th graph show the separation in Ia and Ib volleys Record E shows that a volley in SP can remove that facilitatory action from PBSt Ia afferents at the time relationship between conditioning and testing volleys shown in record E

Curve B is from another experiment with testing of the excitability in Ia afferents from DP A train of volleys in Ia afferents from PBSt gave facilitation to 175 on the ordinate A conditioning volley in G S (stimulus strength 49 times the threshold strength) gives longlasting inhibition of the facilitation but has in itself no effect on the excitability on Ia afferents from DP (×) There was no inhibitory effect by a group I volley from G S

In both curves the abscissa denotes interval between the conditioning FRA volley and the test spike in Ia afferents Chloralose anaesthesia



in record C, the inset records in the curves show the separation in Ia and Ib volleys from PBSt Record D shows the removal of the increased excitability when the PBSt stimulation is preceded by a volley in the superficial peroneal nerve (SP) with the time relationship shown in E The time course of this removal is shown in curve A where 100 is the size of the unconditioned test response and 153 the size of the average facilitation evoked from PBSt Ia afferents There is a characteristic long duration of the depression which corresponds to the long duration of DRP from the SP nerve In this particular experiment the excitability decreased even slightly below the normal test level but record B and the crosses (x) in the curve show that the conditioning SP volley alone has this effect It is probable that in this cat there was a slight tonic primary afferent depolarization in Ia fibres possibly evoked from hip flexor muscles that were not denervated and that the decreased excitability evoked from the FRA is due to an inhibition of this tonic depolarization However in most experiments the FRA did not have any action in themselves as is shown in curve B Fig 7 An additional feature in this curve is that the excitability is tested in Ia afferents from a flexor muscle and that the inhibition is evoked from high threshold muscle afferents

Intracellular recording from Ia afferents The records in Fig 8 are from an experiment with intracellular recording from a Ia afferent of the PBSt nerve D shows the PAD evoked from DP Ia afferents (cf E and H) As expected there is no PAD from the sural nerve (E) but there is an almost complete inhibition of the Ia DRP on combined stimulation of the sural nerve and DP in F The curve in Fig 7 shows the time course of the depression

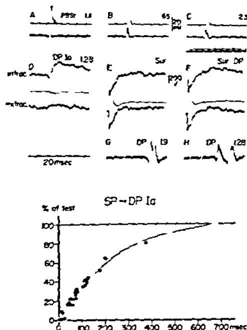


Fig 8 Intracellular recording (KCl electrode) from a PBSt Ia afferent A—C show the spike potential at low amplification in A at threshold for the axon The lower traces are extracellular microelectrode recordings after withdrawal of the electrode to a just extracellular position The lower traces in A—C G H and the middle traces in D—F are recorded from the dorsal root entry zone in L7 The PBSt and DP nerves displayed separation in Ia and Ib volleys A—C and G—H respectively Stimulus strengths are given in multiples of threshold strengths The primary afferent depolarization in A is evoked by Ia afferents from DP (cf record H) The sural volley did not evoke any PAD (E) F shows that on combined stimulation of the sural and the DP nerve there is a complete depression of the Ia PAD from DP The time course of this depression is shown in the graph, where 100 % on the ordinate represent the unconditioned Ia DRP from DP Chloralose anaesthesia

The Ia axon from FDL in Fig 9 receives PAD from PBSt and DP (E—H) but not from G-S (C) and ABSm (D) The effect in F and G is caused by Ia afferents the additional effect in H by Ib afferents When the stimulation strength is raised to include group II (I) and group III (J and K) there is a progressive reduction compared with the PAD evoked in H at maximal group I strength It should be observed that this depression is not only of that component of PAD that is evoked from Ib afferents the size of the remaining PAD in K is much smaller than the maximal Ia PAD in G Hence the high threshold muscle afferents have an inhibitory action on transmission from Ia A similar inhibition from the sural nerve on the Ia DRP is shown at slower speed in L—N

Removal of presynaptic inhibition of transmission to motoneurones

From what has been reported above it would be expected that volleys in the FRA may remove the presynaptic inhibition that can be evoked from Ia afferents of flexors of the Ia EPSP in motoneurones This was regularly found in extensor and flexor motoneurones as is illustrated for a G-S cell in Fig 10 The homonymous Ia EPSP is shown in D A volley in the sural nerve does not change this EPSP (E) F shows the depression that the train of Ia volleys from PBSt gives of the EPSP and in G there is removal of this depression when the sural nerve is stimulated in addition The time relationship of the conditioning and testing volleys is shown in C

Effect of strychnine

The finding that the depression of the Ia DRP from the FRA has the same time course as the DRP evoked from the FRA suggests that there is a presynaptic inhibition oper

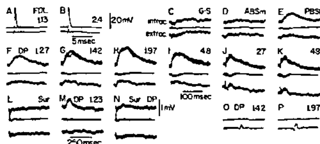


Fig 9 The upper traces in A—N are intracellular (citrate electrode) from a FDL Ia axon The lower traces in C—N were obtained after withdrawal of the microelectrode to a just extracellular position The lower traces in A B O P and middle traces in C—N are from the dorsal root entry zone in L7 Stimulus strengths are given in multiples of threshold strengths The axon receives PAD from group I afferents from PBSt and DP (E and H) but not from G S and ABSm (C and E) F—K are obtained with increasing strength of the DP nerve G being maximal for Ia and H for Ib afferents When the strength is increased above group I there is a marked decrease of the PAD which is apparent already at group II strength in I but more marked in J when the strength is increased to 27 × threshold The test DRP in M is evoked by a train of submaximal Ia volleys. The sural nerve is stimulated in L and in N with combined stimulation of the sural and DP nerve there is an effective depression of the Ia DRP All records consist of superimposed traces Unanaesthetized decorticate spinal cat

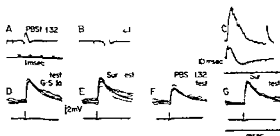


Fig 10 The upper traces in C—G are intracellular (KCl electrode) from a G S motoneurone and the lower traces in those records and records A and B are from the L7 dorsal root entry zone The homonymous test Ia EPSP is shown in D In F there is no effect by a conditioning volley in the sural nerve The depression in F is evoked by a short train of maximal Ia volleys in the PBSt nerve (Ia and Ib volleys shown in A and B) With combined conditioning of the sural and the PBSt nerve there is a removal of the depression in G The time relationship of the conditioning and testing volleys is shown in record C All records except C consist of superimposed traces Chloralose anaesthesia

ating at an interneuronal level through depolarization of terminals of interneurones transmitting effects from Ia to Ia afferents It was therefore of interest to examine the effect of strychnine on this inhibitory action (cf Eccles Schmidt and Willis 1963 a) The FRA depression of the Ia DRP remains unchanged after large doses of strychnine that are sufficient to depress very effectively postsynaptic inhibitory effects from the FRA Experiments with excitability measurements did however reveal the complication that after strychnine volleys in the FRA usually had some action on Ia afferents In most cases it was therefore difficult to exclude that the DRP depression was not at the primary afferent level However in one case we found a complete inhibition of the

Ia PAD from cutaneous afferents without that these afferents had any direct effect on Ia afferents This suggests that the inhibitory effect on transmission from Ia to Ia afferents is not changed by strychnine

Discussion

The present investigation set out from the observation that volleys in the FRA depress the DRP evoked from Ia afferents Depressive interaction between the DRPs from different sources is common but can usually be explained by the effects that these afferents have on primary afferents (Eccles Kostyuk and Schmidt 1962 Carpenter Lundberg and Norrsell 1963) The depression from the FRA of the Ia DRP could be exerted at the primary afferent level only if volleys in the FRA and in Ia afferents evoked PAD in the same primary afferents Under special conditions such an action may be found (Andén *et al* 1964) but there is no evidence that this occurs in the normal acute spinal cat We have confirmed that volleys in the FRA act on the FRA and also on Ib afferents but not on Ia afferents and that volleys in Ia afferents from flexors evoke PAD in Ia afferents but not in Ib afferents and the FRA (Eccles Magni and Willis 1962 Eccles *et al* 1963 b c Fig 3—6 above) Hence it was possible to formulate the working hypothesis that volleys in the FRA depress transmission from Ia to Ia afferents at an interneuronal level

However the problem could clearly not be solved with investigation of DRPs in particular there is the possibility that the FRA and Ia afferents may act together on some afferent system hitherto not investigated It was therefore necessary to investigate directly the Ia afferent pathway and this has been done with excitability measurements ad modum Wall (1958) and with intracellular recording from Ia afferents With both these methods it has now been shown that volleys in the FRA prevent the establishment of the PAD from Ia in Ia afferents Correspondingly volleys in the FRA are very effective in removing the presynaptic inhibition that Ia afferents from flexors give of the Ia EPSP in motoneurones (Frank and Fourtes 1957 Eccles *et al* 1961) Since volleys in the FRA do not evoke PAD in Ia afferents it is postulated that this inhibition from the FRA occurs on the interneurones transmitting effect from Ia to Ia

The time course of the inhibition is practically identical with the time course of the PAD evoked from the FRA This raises the question if the inhibition is presynaptic through depolarization of interneuronal terminals The inhibition survives large doses of strychnine but experiments with strychnine are not decisive since it recently has been shown that longlasting postsynaptic inhibitory pathways at higher levels are not affected by strychnine (Andersen *et al* 1963) and there is no reason why this could not occur also in the spinal cord

It has already been reported that a similar depression of transmission to Ia afferents can be evoked from the sensorimotor cortex (Lundberg and Vyklicky 1963 The corticospinal tract acts on the segmental level through excitation of interneurones of reflex paths (Lundberg and Voorhoeve 1962 Lundberg Norrsell and Voorhoeve 1962 Carpenter Lundberg and Norrsell 1963 Engberg 1964 Hence it is assumed that the depression from cortex on transmission from the FRA is caused by excitation of interneurones mediating this inhibitory action from the FRA

It is at present difficult to appreciate the functional significance of presynaptic inhibition of Ia actions *It seems more easy to understand the functional role* that its removal may have In the flexor reflex there is also activation of γ motoneurones Hunt

1951 Hunt and Paintal 1958 Voorhoeve and van Kanten 1962) In activation of the FRA the removal of an existing PAD in Ia afferents will give optimal function in the pathway from Ia afferents to α motoneurones and hence provide for the γ loop to be maximally effective

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The time course of the inhibition is practically identical with the time course of the PAD evoked from the FRA This raises the question if the inhibition is presynaptic through depolarization of interneuronal terminals The inhibition survives large doses of strychnine but experiments with strychnine are not decisive since it recently has been shown that long-lasting postsynaptic inhibitory pathways at higher levels are not affected by strychnine (Andersen *et al* 1963) and there is no reason why this could not occur also in the spinal cord

It has already been reported that a similar depression of transmission to Ia afferents can be evoked from the sensorimotor cortex (Lundberg and Vyklicky 1963) The corticospinal tract acts on the segmental level through excitation of interneurones of reflex paths (Lundberg and Voorhoeve 1962 Lundberg Norrsell and Voorhoeve 1962 Carpenter Lundberg and Norrsell 1963 Engberg 1964 Hence it is assumed that the depression from cortex on transmission from the FRA is caused by excitation of interneurones mediating this inhibitory action from the FRA

It is at present difficult to appreciate the functional significance of presynaptic inhibition of Ia actions It seems more easy to understand the functional role that its removal may have In the flexor reflex there is also activation of γ motoneurones (Hunt

TABLE I The effect of cortisone on the urinary excretion of total hydroxyproline (μg/24 hours mean±SD) and on the weight (grams mean±SD) of 2 and 7 month old rats

Age (months)	Group	Weight	Total hydroxyprolin
2	Controls 0	156±22	474±150
	VIII	187±14	510± 76
	Cortisone 0	150±17	500±157
	VIII	164±18	362± 83
7	Controls 0	302±18	320± 62
	VIII	306±23	358± 38
	Cortisone 0	310±29	349± 32
	VIII	288±26	339± 36

P<0 01

6 rats in both groups of 2 month old rats and 7 rats in both groups of 7 month-old rats
Daily dose of cortisone acetate 3 mg in 2 and 6 mg in 7 month-old rats
0 initial value VIII=after 8 injections

catabolism of the insoluble collagen fibres (Lindstedt and Prockop 1961 Prockop 1964) It therefore seemed possible that in young animals the decrease in the content of soluble collagen fractions caused by cortisone would be reflected as a decreased excretion of hydroxyproline in the urine although such an effect has not been found in older animals

Material and methods

The experimental animals were male albino Wistar rats 2 and 7 months of age They were fed ad libitum with a commercial pelleted diet (Hankkija Oy) and allowed free access to water

Cortisone acetate (Adreson Organon Oss) was injected intraperitoneally The 2 month-old rats received 3 mg and 7 month-old rats 6 mg of cortisone acetate daily Urine was collected during a 14 hr period at the beginning of the experiment and after 8 injections and in the 2 month-old rats after 3 injections also The values were then converted to μg per 24 hrs The animals were killed 2 hrs after the 9th injection after a fasting period of 16 hrs and the blood samples were taken

The urine samples were analysed for total hydroxyproline and the samples taken after 8 injections for free hydroxyproline also After deproteinization with trichloroacetic acid the serum samples were analysed for free hydroxyproline Hydroxyproline was determined by the method of Prockop and Udenfriend (1960)

Results

The administration of cortisone caused a small delay in the weight gain of the 2 month old rats and some loss of weight in the 7 month-old rats (Table I) The initial excretion of total hydroxyproline in the urine was considerably greater in 2 month-old than in 7 month-old rats and there was a significant decrease in the excretion of total hydroxy

TABLE II The effect of cortisone on the urinary excretion of free hydroxyproline (μg/24 hours mean±SD) and on the concentration of free hydroxyproline in the serum (μg/ml mean±SD) in 2- and 7-month-old rats

Age (months)	Group	Free hydroxyproline in the urine	Free hydroxyproline in the serum
2	Controls	90.2±19.5	3.93±0.61
	Cortisone	[1]153.7±34.6	[2]3.05±0.27
7	Controls	49.7±18.7	1.49±0.15
	Cortisone	38.8±15.2	1.35±0.14

[1] P < 0.05 [2] P < 0.01
6 rats in both groups of 2-month-old rats and 7 rats in both groups of 7-month-old rats
Daily dose of cortisone acetate 3 mg in 2- and 6 mg in 7-month-old rats. The urinary values were analysed after 8 injections and the serum values after 9 injections

proline in the cortisone-treated 2-month-old rats but no change in the older rats (Table I). The excretion of total hydroxyproline in 2-month-old rats was also analysed after 3 injections but at this stage of the experiment there was as yet no difference between the excretion of the cortisone-treated rats and the controls.

The excretion of free hydroxyproline in the urine and the concentration of free hydroxyproline in the serum at the end of the experiment are shown in Table II. These values, like the values of total hydroxyproline, were considerably higher in the 2-month-old controls than in the 7-month-old ones. Administration of cortisone significantly decreased both the urinary excretion of free hydroxyproline and the concentration of free hydroxyproline in the serum in the 2-month-old rats. In the 7-month-old rats the mean values were likewise slightly decreased in the cortisone-treated animals but the decreases were considerably smaller than in the 2-month-old rats and not statistically significant.

Discussion

According to the present view collagen fibres are built up from tropocollagen molecules that are synthesized intracellularly and combine in the extracellular space to form the insoluble collagen fibres. The content of the soluble collagen fractions, i.e. of fractions containing collagen not yet aggregated into insoluble fibres, in the tissues of animals (e.g. Nageotte and Guyon 1934, Orekhovič 1950, Kobrle and Chvapil 1958) and the excretion of hydroxyproline in the urine (e.g. Ziff *et al.* 1956, Kivirikko and Liesmaa 1958, Lindstedt and Prockop 1961) decrease during the aging of animals. Experiments with C-proline indicate that a considerable part of the hydroxyproline in the urine of young rats is derived from the soluble collagen fractions but some catabolism of the insoluble collagen fibres to urinary hydroxyproline also occurs. In older rats a relatively smaller proportion of the urinary hydroxyproline is derived from the soluble collagen fractions than in the young ones and a relatively greater part from the catabolism of the insoluble collagen fibres (Lindstedt and Prockop 1961, Prockop 1964). Studies on

the effect of age growth scurvy lathyrism and thyroid hormones on the excretion of hydroxyproline in the urine have likewise indicated that urinary hydroxyproline reflects changes in body collagen and especially in the soluble collagen fractions (Ziff *et al* 1956 Martin Mergenhagen and Prockop 1961 Jasin and Ziff 1962 Jasin *et al* 1962 Kivirikko *et al* 1963 Kivirikko Koivusalo and Laitinen 1964)

It therefore seemed surprising that the administration of cortisone was found not to cause any change in the urinary excretion of hydroxyproline (Ziff *et al* 1956 Kivirikko and Liesmaa 1958) although it decreases the content of soluble collagen fractions in the skin of animals (*e g* Siuko Savela and Kulonen 1959 Sakata 1960 Gunther and Carsten 1961 Sethi Ramey and Houck 1961) The results of the present study indicate that the concentration of free hydroxyproline in the serum and the excretion of free and total hydroxyproline in the urine decreased considerably in 2 month-old rats after the administration of cortisone By contrast in 7 month-old rats which had a considerably lower basal concentration of free hydroxyproline in the serum and basal excretion of free and total hydroxyproline in the urine no changes or only slightly decreased values were observed after cortisone administration

These results can be explained on the basis of the studies with C-proline cited above (Lindstedt and Prockop 1961) The decrease in the content of soluble collagen fractions known to occur after the administration of cortisone was reflected in a decreased excretion of hydroxyproline in 2 month-old rats In 7 month-old rats considerably smaller amounts of urinary hydroxyproline were derived from the soluble collagen fraction and a relatively greater part from the catabolism of the insoluble collagen fibres Therefore the decreased excretion of hydroxyproline derived from the soluble collagen fractions caused only a very small reduction in the excretion of hydroxyproline in the urine of the older animals

The origin of free hydroxyproline in the serum has been studied less The concentration of free hydroxyproline in the serum like the excretion of hydroxyproline in the urine decreases during the aging of animals (Kobrle and Chvapil 1961) and increases after the administration of growth hormone (Kivirikko Liesmaa and Luukkainen 1958) in hyperparathyroidism (Bates, McGoven and Talmage 1962) and in hyperthyroidism (Kivirikko *et al* 1964) In the present study the concentration of free hydroxyproline in the serum decreased considerably in 2 month-old rats but only very slightly in 7 month-old rats after the administration of cortisone Thus it seems probable that the free hydroxyproline in the serum like the hydroxyproline in the urine has its origin both in the soluble collagen fractions and in the insoluble collagen fibres and that a considerably greater part of the hydroxyproline in the serum is derived from the soluble collagen fractions in young animals than in older ones

Different opinions have been expressed in the literature concerning the mode of the inhibitory action of cortisone on the metabolism of collagen Several studies indicate an antianabolic effect (*e g* Chvapil 1959 Siuko Savela and Kulonen 1959 Sakata 1960 Mazurov and Orehović 1960 Kivirikko 1963 Ebert and Prockop 1963 but some studies suggest that cortisone in addition increases the catabolism of the collagen fibres (*e g* Sakata 1960) The results of the present study support the hypothesis that the effect of cortisone on the metabolism of collagen is mainly antianabolic and not catabolic for a catabolic action would increase and not decrease the excretion of hydroxyproline in the urine

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Ionic Accumulation by Water Flow through a Membrane

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Abstract

Rapoport, S. I. *Ionic accumulation by water flow through a membrane.* Acta physiol. scand. 1965. 64. 361–371. — The relation between diffusion and water flow for solutions of KCl and of KCl + LiCl was studied in the absence of electric current on artificial wide pore membranes which were effectively uncharged and nonselective. Ionic mobilities in the membrane were obtained from limiting ionic conductances in water. Diffusion coefficients for KCl in the membrane were obtained under zero and non zero flow conditions. They were used to compare predicted with observed steady state specific ionic and accumulation ratios. Significant disagreement was found only between the diffusion coefficient derived from the observed accumulation ratios and the zero flow coefficient. Possible reasons were discussed. An accumulation ratio of 6.9 for K^+ to Li^+ was obtained. In the non steady state the flux of KCl when the sole solute could be represented by a proposed equation (Eq. 4). With KCl + LiCl the net cationic flux ratio could also be predicted (Eq. 8). At the steady state the system represented a first order state in which the force acting on water was constant and in which a transport mechanism which was not ion specific existed.

An important problem in biology concerns the metabolically supported accumulation of K with respect to Na within many types of cells in the face of a reversed distribution of these ions in the extracellular fluid. In certain cases the concentrating ability of the cell is thought to depend on active ion-specific transport mechanisms (reviewed by Woodbury 1960). This paper will consider on the other hand the possible importance of water flow as a factor in ionic accumulation.

A cell model was constructed consisting of two compartments separated by a membrane in which gradients of hydrostatic pressure and of concentration could be produced. K and Li were chosen as representative ions because of the difference between their mobilities (Robinson and Stokes 1955, p. 46).

Three membrane factors affecting ionic accumulation will be discussed: (1) charge and selectivity, (2) ionic mobility within the membrane, (3) water flow effect. In addition, steady state accumulation as well as the net flux ratio in the non-steady state will be analyzed.

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Theory

The mathematical principles of accumulation were derived by Hertz (1922 1923) for gases and by Teorell (1951) for ions in solution Other treatments have been published (Manegold and Solf 1932 Williams and Cady 1954 Bloch 1946 Koefoed Johnsen and Ussing 1953 Nims and Thurber 1961 Ekman Rastas and Salminen 1963) The Hertz formula applied to solutions has been verified experimentally (Garby 1957) and the Teorell formulation has been analyzed also (Nims and Thurber 1961 Ekman *et al* 1963)

Net Flux

The fundamental equation for flux (moles/sec/total membrane area) along the x axis perpendicular to the plane of the membrane and in the positive direction (Fig 1) is for the cation (Teorell 1953)

$$\phi = - RTu\ C\ \frac{d\ln(\eta\ \xi C)}{dx} \tag{1}$$

where R = gas constant (joules/degree/mole) T = absolute temperature u^+ = mobility of specific ion in the total membrane area (under unit chemical potential gradient) C = cation concentration $\xi = \exp(F\psi/RT)$ ψ = membrane potential (volts) F = Faraday (coulombs) η exp$(-vx/RTu^+)$ (a specific ionic water function) v = total bulk flow through the membrane in a positive direction (ml/sec) (where v/membrane area = water velocity cm² membrane)

When flux is constant Eq 1 may be integrated partially (Sandblom unpublished) letting and ψ 1 at x 0

$$\phi \quad RTu\ \frac{(C\ \xi\ \eta_x - C\ )}{_0\int^1 \eta\ dx} \tag{2}$$

For a single salt (KCl for example) when electric current 0 the flux of K through the membrane equals that of Cl Eq 2 reduces to an integrated form of the Hertz equation where is the apparent mobility of both K and Cl in the membrane

$$I \quad \frac{(C \quad \phi_t RT \quad C_1)}{(1 - \quad vd\ RT \quad)} \tag{3}$$

Here I membrane thickness(cm) For the case C 0 Eq 3 has been derived (Manegold and Solf 1932) and verified (Garby 1957)

When C_1 and are constant Eq 3 is a first order differential with C_2 as a function of time t since ϕ V (dC /dt) (where V is the volume of compartment 2) In a thin membrane if the denominator and numerator of Eq 3 are multiplied by exp(vd RTu) the denominator will approximate vd RTu so that the equation may be integrated to give (if membrane concentration profiles do not change rapidly)

$$C \quad C \quad [1 \quad \exp(-RTu \quad t\ d)] \tag{4}$$

Ion Accumulation

For zero current flux Eq reduces to (Teorell 1951)

$$C \quad C \quad \xi\ \exp\ d\ RTu \tag{5}$$

The prime refers to any specific ion species

If another flux also zero a relation similar to that of Eq 1 be obtained for the anion where ξ is replaced by and u is replaced by u For a solution of two univalent electrolytes with a common anion (KCl NaCl for example) the anion ratio C_2^{Cl}/C_1^{Cl} may be multiplied by either of the two cation ratios In an uncharged membrane relative ionic mobilities should be the same as in water (see below) and use of Eq 10 (see below) will permit

derivat on of a flow independent expression of Nims and Thurber (1961) shown to be val l for a solution of NaCl + KCl

$$\frac{\ln (C_2^{Li} C_2^{Cl}/C_1^{Li} C_1^{Cl})}{\ln (C^{K} C^{Cl}/C_1^{K} C_1^{Cl})} = \frac{D^{KCl}}{D^{LiCl}} \cong 1\ 45 \tag{6}$$

D^{KCl} and D^{LiCl} are the respective d ffusion coeff cients of KCl and LiCl within the mem brane

For two diffusing species (K^+ and Li^+ for example) water flow counter to d ffusion should induce accumulat on of the species *with the higher mobility* on side 2 of the membrane (Teorell 1951) The *accumulati n rati* A at the steady state is gi en from Eq 5 b

$$A = \frac{C^{K}/C_1^{K}}{C^{Li}/C_1^{Li}} = \exp\left[\frac{vd}{RT}\left(\frac{1}{u^{K}} - \frac{1}{I}\right)\right] \tag{7}$$

Eqs 5 and 7 must be mod fied for charged membranes (Teorell 1951) and Eq 5 should in clude activity coefficients for greater accuracy (coeff cients cancel from Eq 7)

Flux R tio

The *n t flux rat* of K^+ to Li^+ may be obtained from the quoti nt of the individual fluxes each gi en by Eq 2 and has been derived by Behn (1897) for $v = 0$ When $v \neq 0$ the membrane p tential and concentrat on profiles must be kno n in o der to calculate the flux ratio The mean value theorem of the integral calcules ndicates that for and η^+ continuous in the membrane and η 0 the integral term in the denom nator of Eq 2 may be replaced by $\int_0^d$ dx where is s me value of ξ in the membrane Then division of the K flux by the L flux (Eq 2) g s th *net flux ratio*

$$\frac{\phi^{K}}{\phi^{Li}} = S\ \frac{(C_2^{K}\ \xi_2\ ^{K} - C_1^{K})}{(C_2^{Li}\ \eta_2^{Li} - C_1^{Li})}\ \frac{(1 - \eta^{Li})}{(1 - \eta^{K})} \tag{8}$$

where S = Li K Eq 8 w ll be test d letting $S = 1$

Experiments were designed to test the validity of Eqs 3 to 8

Methods

A plex glass chamber cons sting of t o compartments separated b a membrane was imm rsed in a water bath at 25 ± 0 1 C (F g 1) Solutions (10—?0 ml in ach compartment) were stirred by vibrating glass paddles

Th conc ntration of solute in Compartment 1 [e ther about 0 015 M KCl + 0 015 M L Cl (Expts 1—10) or about 0 075 M KCl + 0 075 M LiCl (E pt 11 12) or about 0 03 M KCl (Expts 13—17)] was held onstant f r th durat on f th expe ment by flowing a solu ti n f id nt cal solute concentrat on nto t and by mainta ning th pressure P con tar t by overfl w dra nag P was k pt at 1P dynes cm abo P by dr pp ng distilled water t C mpartment at a constar t at (— e ml ec) through a f n bo r glas capillary n ler a f xed pres u e h ad The c nc ntration of solut n Compartment 2 C was 0 at th beg n ning of each experiment an l ncreased with t me to a teady tate lev l

A i gle xperiment cons ted n measuring the appearanc of solute n Co mpa tment 2 The concentrat on of a ngl alt (KCl f exampl) was d term n d by m as ring the sistanc betwe n 2 p evi u ly tandard zed Pt black Pt lectr des imm rsed in Comp rtment 2 (1 000 c p s Conductol zer Type 5300 B LKB Stockh lm) For two salts (KCl + LiCl) th res tance nd cated hanges n total oncent tion Samples 0 5 t 5 ml) were w thdrawn peri dically f m Compartm nt 2 an l r pl c d with eq i al nt l mes f d t ll l water dur ng a run and th ind lual n c c ncentrat ns wer d term ned later by m ans f flame ph t m try Th peri d mpl ng procedu was f ll wed als n th few perim nts with

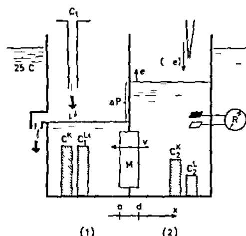


Fig 1 *Experimental compartmental system illustrated with KCl + LiCl at the steady state* Concentrations in Compartment 1 (C_1's) are kept constant by a high rate of circulation of solution at C_1 C_2 variable A pressure difference ΔP is maintained by overflow drainage in Compartment 1 and by dripping of distilled water into Compartment 2 at $(v + e)$ ml/sec Positive direction from left to right along x axis Resistance between 2 Pt black Pt electrodes can be followed in Compartment 2 M = membrane d = membrane thickness a = membrane surface area e = estimated evaporation rate from Compartment 2 $-v$ = total water flow through membrane in negative direction The figure shows relative accumulation of K^+

glucose (cf p 365) whose concentration was determined by the anthrone method of Wallenius (1954)

Three types of cellulose derivative membranes were used Membrane (A) was a single Group 5 membrane (Membrane Filter Gottingen) $d = 0.008$ cm (by micrometer measurement dry state) average pore diameter $= 0.26\,\mu$ Membrane (B) was a Group 3 Membrane Filter $d = 0.013$ cm average pore diameter $= 0.3\,\mu$ Membrane (C) consisted of three Group 1 Membrane Filters which were compressed together by two plastic walls with six overlapping holes total $d = 0.046$ cm average pore diameter $= 0.6\,\mu$ Membrane surface for diffusion a ranged from 2 5 to 3 5 cm with the pore area composing about 60 % (see below)

In some experiments membrane potential was measured by means of two matched calomel electrodes and a high impedance volt meter

Experimental Procedures

An experiment consisted in determining first the diffusion coefficient of KCl in the membrane D^{KCl} for the case $\Delta P = 0$ and $v = 0$ After the system had run for 1/2 hr ($C_1^{KCl} = 0.03$ M $C_1^{LiCl} = 0$) the initial condition ($C_2^{KCl} = 0$) was set and C_2^{KCl} was determined periodically The volume V of Compartment 2 was determined at the end of an about 3 hr run by spectrophotometric measurement (at 510 and 520 mμ) of the dilution of a known quantity of $CoCl_2$ (1 ml 0 75 M in 3 HCl) which had been introduced into Compartment 2 D^{KCl} assumed constant over the concentration range examined could be calculated then from Fick's First Law with a S D of 2 8 as follows

$$D^{KCl} = Vd\frac{d\ln[(C_1 - C_2/C_1)]}{dt} \tag{9}$$

For C_1^{KCl} = Constant plotting the numerator on the right side of Eq 9 against time gave an approximate straight line with slope $= D^{KCl}$ (a cm /sec)

Water Flow After v for the experiments was determined by weighing the water coming from the fine glass capillary and by correcting for evaporation (Fig 1) ΔP was adjusted from known flow pressure characteristics of the membrane The system was allowed to run for about 12 hrs with distilled water in both compartments so as to obtain exact flow pressure equilibrium and then the water in Compartment 1 was replaced with rapidly circulating solution at C_1

The experiment proceeded for 3 to 5 days while periodic samples were taken and then final concentrations in both compartments were found and v was redetermined The mean of the

in tial and final values of r (which changed by an average of 4 during a run) was used in calculations I was also determined

D^{KCl} which changed by an average of 6 % during a run was determined at the end of the run the mean of its initial and final values was used for calculation Changes in D^{KCl} may have been due to blocking of membrane pores (Nims and Thurber 1961) to chemical changes in the membrane or to changes in stirring efficiency Although such changes would be associated with changes in membrane resistance to water flow the model was arranged so that flow remained essentially constant during a run (Fig 1)

Membrane Factors

1 *Cha e and Select ity* A membrane with pores >0 1 μ in diameter should not be selective for univalent ions (Bikerman 1958 p 440 Sollner 1945) so that *relative* ionic mobilities in the membrane should be the same as in aqueous solution

The absence of selectivity in Membranes (A) and (C) was suggested by finding only a small (< 0 001 N) apparent negative charge when membrane potential was measured for a single salt system when C_2^{KCl} ranged between 0 01 and 1 0 M and when $C_2^{KCl}/C_1^{KCl} = 10/1$ (Teorell 1953) A surface water film may have affected the results (Helfferich 1962 p 26?)

The membranes did not select charged from low molecular weight uncharged molecules the apparent pore area of Membrane (A) for KCl or for glucose diffusion was respectively 20 7 ± 2 7(S D) and 20 4 ± 0 7(S D) of the membrane surface (cf Garby 1957) Pore area was calculated by di d ng the d ffusion coefficient within one membrane by the respective coefficient in water (Robinson and Stokes 1959 p 466 Hodgman 1962 p 2275) if a path length of $3d$ rather than d were used in the calculations (Manegold and Solf 1932) pore area would approximate 60

2 *I n M bility withi the Membrane* The diffusion coefficient of a dilute univalent salt may be derived from the l m ting conductances (λ) of its ions (Robinson and Stokes 1959 p 288)

$$D = \frac{2RT}{F^2}\frac{\lambda_\infty \lambda_x}{\lambda_\infty \lambda_x} \tag{10}$$

The limiting ion c mobility is $u = \lambda_x/F^2$ RTu which equals $RT\lambda$ $/F^2$ may be called the diffusion coefficient of the single ion ( ) Dividing the membrane diffusion coefficient of KCl D^{KCl} by the ionic diffusion coefficient gives a specific ionic *mult plyi facto f* whose value may be obtained from limiting ionic conductances (Conductance ratios among the three species K^+ Li^+ and Cl^- do not differ by more than 2 from limiting conductance ratios in solutions of ionic strength of 0 01 / — about 1/3 of the ionic strength of Compartment I in all but expts 11 and 12 (Kell and Gordon 1959))

Thus the K^+ mult plying factor is (D from Eq 10)

$$f^K = D \Big/ \frac{RT\lambda_x^K}{F^2} \tag{11}$$

The factors ar for K^+ Li^+ and Cl respectively at 25 C 0 93 0 517 and 1 0 using data from Robinson and Stokes (1959 p 465) Each factor will permit calculation of the diffusion coefficient of the respective ion and thus of the specific ionic mobility in the membrane

3 *Water Flow Eff ct Calculat o f Membrane P tent l* The potential across Membrane (A) and Membrane (B) was measured when the total concentrations of solutions equimolar (0 015 M) in KCl and LiCl ranged from ratios (C C) of 1 10 to 1 1 across the membrane and when ncluded all values in Table I (see below) 10 decrease in the absolute value of the zero flow potential occurred when was very negative and when at the same time the concentration ratio was low Correspondingly only expts 1 and 7 (Table I) should have had more than a 10 deviation in the zero flow potential a deviation which may have been an electrokinetic effect (Schmid and Schwarz 1952)

Measured zero flow potentials were found to equal diffusion potentials calculated by the Henderson equation (MacInnes 1939 p 231 Therefore for experiments in Table I φ was obtained by the Henderson equation to calculate and should have been correct to within 10 in all but Expts 1 and 7 In Expt 1 to 12 φ was calculated to be between — 1 and — 14 mV

TABLE I *Results of flow experiments* Expts 1—12 were done with KCl + LiCl and 13—17 were done with KCl D_o^{KCl} (a cm /s c × 10 ⁴) is the zero flow diffusion coefficient of KCl in the membrane (Eq 9) A_{pred} is the accumulation ratio predicted from the right hand side of Eq 7 using D_o^{KCl} A_{obs} is the experimentally observed accumulation ratio at the steady state from which an observed value for the diffusion coefficient of KCl D_A^{KCl} (a cm /sec × 10) was obtained by solving Eq 7 $(C_2/C_1)_{obs}$ is the experimentally observed steady state specific ionic ratio From the respective K and Li ratios with insertion of ξ ¹ into Eq 5 the coefficients D_K^{KCl} and D_{Li}^{KCl} (a cm /sec × 10 ⁴) could be obtained Neither D_K^{KCl} nor D_L^{KCl} differed significantly from D^{KCl} for Expts 1—12 but D_K^{KCl} was greater than D_o^{KCl} ι = membrane water flow (ml/sec × 10) d = membrane thickness (cm × 10 ⁴) a = membrane surface area (cm)

Expt no	D_o^{KCl}	ι	d	A_{pred}	A_{obs}	D_A^{KCl}	C_2^K/C_1^K	C_2^L/C_1^L	ξ	D_K^{KCl}	D_L^{KCl}
1	0 774	−13 1	0 8	3 5	2 2	1 2	0 361	0 164	1 ?8	0 86	1 0
2	0 752	−4 05	0 8	1 5	1 5	0 77	0 643	0 437	1 12	0 59	0 67
3	0 751	−8 99	0 8	2 4	1 7	1 2	0 510	0 303	1 17	0 88	1 0
4	1 41	−3 15	0 8	1 2	1 2	1 3	0 881	0 739	1 04	1 5	1 4
5	1 42	−4 08	0 8	1 2	1 2	1 6	0 858	0 713	1 04	1 7	1 7
6	1 53	−4 63	4 6	3 6	3 2	1 7	0 340	0 107	1 32	1 6	1 7
7	1 31	−9 17	4 6	19	6 9	?0	0 055	0 008	1 73	1 3	1 5
8	1 10	−1 26	4 6	1 6	1 7	1 0	0 534	0 305	1 17	0 75	0 65
9	1 81	−3 26	4 6	2 1	1 9	2 1	0 [illegible]03	0 262	1 19	1 8	1 [illegible]
10	1 87	−1 07	4 6	1 3	1 3	1 8	0 813	0 628	1 07	1 8	1 8
11	1 32	−7 70	1 3	2 0	1 7	1 7	0 610	0 357	1 14	1 6	1 7
12	1 56	−8 46	1 3	1 9	1 5	2 4	0 559	0 366	1 14	1 6	1 [illegible]
13	0 [illegible]00	1 83	0 8				0 811			0 70	
14	0 760	−3 44	0 8				0 687			0 73	
15	1 31	−3 18	0 8				0 805			1 2	
16	1 42	−4 09	0 8				0 788			1 4	
17	0 751	−9 08	0 8				0 500			1 0	

Results

I Accumulation Equations 5 and 7

For each of 12 experiments with KCl LiCl (Table I) the value of the *predicted* accumulation ratio was calculated from the right hand side of Eq 7 letting $RTu^K = f^K D_o^{KCl}$ and $RTu^{Li} = f^L D^{KCl}$ cf Eq 11 The quotient A_{pred}/A_{obs} of the predicted to the *observed* (left hand side of Eq 7 accumulation ratio was found for each experiment The mean of the quotients calculated from the *ln* values of the quotients was 114 ± 6(S E) ($P <$ 0 05) that mean differs from 100 Expt 7 was excluded

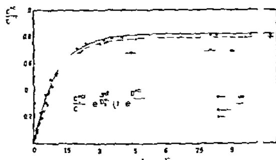


Fig. ? Entry of KCl into Compartment ? $C^{KCl} = 0$ at $t = 0$, $C_1^{KCl} = 0.03$ M Expt. No refers to Table 1 Observed values of C^{KCl} follow the curves predicted by Eq. 4 ($RT\bar{\omega} = D_o^{KCl}$). Expt. 17 which is not included in the figure can be rejected at the $P = 0.05$ level

by the χ^2 distribution of Dixon and Massey (1957 p. 276). Thus A_{pred} was about 10% greater than A_{obs}.

The steady state specific ionic ratio C'/C_1 (left hand side of Eq. 5) was found experimentally for each cation species. Values for σ (calculated by the Henderson formula), r, d and f were placed in Eq. 5 which was then solved for the "apparent" diffusion coefficient of KCl in the membrane under flow conditions. Coefficients derived from the observed K ratios will be called D_K^{KCl} and those derived from the observed Li ratios will be called D_{Li}^{KCl}. Similarly an observed diffusion coefficient could be derived from the accumulation ratios of Expts. 1—12 by use of Eq. 7 and will be labeled D_A^{KCl}.

Each of the diffusion coefficients thus derived from observed ionic ratios at the steady state was compared with D^{KCl} (cf. Eq. 9), the zero flow diffusion coefficient of KCl in the membrane. A comparison of diffusion coefficients seemed more valid than one of predicted and observed concentration or accumulation ratios because an error in a coefficient would appear in the exponential terms of Eqs. 5 and 7 and would distort the ratios at high flow rates (see A_o vs. Exp. 7 Table I).

The quotients D_K^{KCl}/D_o^{KCl}, D_{Li}^{KCl}/D_o^{KCl} and D_A^{KCl}/D^{KCl} were calculated for each two salt experiment (1—12) and ln values of the quotients were used to calculate quotient means in per cent units of D^{KCl}. The means were: for D_K^{KCl}—101 ± 3 (S.E.) ($P > 0.05$ that mean differs from 100); for D_{Li}^{KCl}—109 ± 5 (S.E.) ($P < 0.05$) and for D_A^{KCl}—121 ± 8 (S.E.) ($P < 0.05$).

A low estimate of f^{Li} might explain why D_A^{KCl} was significantly greater than D_o^{KCl} and why $D_{Li}^{KCl} > D_K^{KCl}$ in 9 of 12 experiments. D_A^{KCl}/D^{KCl} was correlated significantly with water flow $\bar{v}$ ($r = -0.918$, $P < 0.05$).

Equation 6

Data given in Table 1 were used to calculate the value of the left hand side of Eq. 6 which is invariant with flow. For expts. 1—12 the mean of this value was 1.42 ± 0.03 (S.E.), not significantly different ($P > 0.05$) from the predicted value of 1.4+

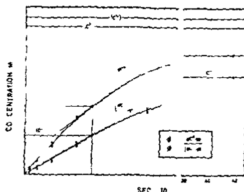


Fig 3

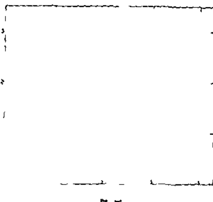


Fig 4

Fig 3 *Influx of K+ and Li+ into Compartment 2* Expt 10 Table I Each pair of points represents C before (higher point) and after sampling C_1 kept constant The "observed flux ratio is the ratio of the tangents of each of the curves at time τ The water flow terms (η s) are calculated from steady state concentrations and from the value of ε (obtained from the Henderson equation) The water flow terms and the values (C^K) and (C^{Li}) are placed in the right hand side of Eq 8 to obtain a predicted flux ratio at time τ

Fig 4 *Relation of observed to predicted net flux ratios* Results of 9 experiments The observed" ratios I K/I Li were determined graphically (method of Fig 3) at 2 to 4 widely separated instants during a single experiment The predicted ratios were calculated from the right hand side of Eq 8 letting S l c = 1 The mean of the differences Robserved Rpredicted did not differ significantly (P 0 05) from zero

Time Flux with Time Equations 3 and 4

Table I summarizes the results of 5 flow experiments (13—17) with KCl alone For these experiments the observed D^{KCl}_K did not differ significantly (P > 0 05) from D^{KCl} and the steady state cationic ratio did not differ significantly from the right hand side of Eq 5 (letting ξ = 1 and $RTu^k = D^{KCl}_0$) Moreover the appearance of KCl in Compartment 2 followed the exponential course given by Eq 4 (Fig 2)

Net Flux Ratio Equation 8

The concentrations of Li and K in Compartment 2 determined periodically during a flow experiment were plotted against time (see Fig 3 for example The ratio of the tangents to each of the curves at a specific instant τ was the *observed* flux ratio at that instant (left hand side of Eq 8)

From the instantaneous concentrations (C^l) and (C^k) the value of ε was calculated from the Henderson equation noting again the possible error for high flows From steady state equilibrium conditions η^k and η^l and thus D^{KCl}_K and D^{KCl}_l were calculated noting that these observed diffusion coefficients did not differ from D^{KCl} The values were inserted into the right hand side of Eq 8 together with figures for c d f^k and f^l

Letting S 1 as a first approximation the flux ratios calculated from the right hand side of Eq 8 were compared with those obtained from observed tangent ratios (Fig 3) for 2 to 4 widely separated instants per experiment

The predicted flux ratios did not differ significantly ($P > 0.05$) from the observed ones and Fig 4 shows the relation between them Thus knowledge of the steady state (final) conditions for a flow experiment with two salts or of D_0^{KCl} will permit calculation by Eq 8 of the net flux ratios for any values of C_2^{K} and C_2^{Li} at any instant during the experiment

The accord between the predicted and observed net flux ratios depended on the fact that in Eq 8 the error in ξ which arose because φ was calculated from the Henderson equation for $v = 0$ was unimportant (p 365) When $C \gg C$ so that the error was maximal ξ canceled out from the right hand side of Eq 8 When $C_1 = C$ the error in ξ was small, since φ deviated not more than 10 from the ($v = 0$) value

For a flow experiment with $C_1^{K} = C_1^{Li}$ $C_2 = 0$ initially the accumulation ratio (defined by the left hand side of Eq 7) was maximal and equal to the net flux ratio initially and then decreased with time to a steady state minimum (Fig 3)

Discussion

The results demonstrate that water flow can produce ionic accumulation in a two compartment model system according to the formulations of Hertz (1922 1923) and Teorell (1951) A hydrostatic pressure difference across a membrane having little resistance to bulk flow (a wide pore membrane) can lead to significant concentration gradients of K Li and Cl at the steady state Each ionic ratio depends on the ratio of bulk flow v to ionic mobility u as well as on electrical potential Accumulation should be independent of potential and theoretically may be made as high as desired for ions whose mobilities differ

Validity of Equations

The relevant equations for the specific ionic ratio (Eq 5) and for ionic accumulation (Eqs 6 and 7) were tested by comparing the zero flow diffusion coefficient D^{KCl} of KCl within the membrane (cf Eq 8) with the coefficients derived from experimentally observed values of ionic concentration and from cell and membrane constants Eq 5 was confirmed when membrane potential was calculated from the Henderson equation for diffusion potential Eq 6 also was reconfirmed

The prediction (Eq 7) that accumulation should be independent of membrane potential was not tested adequately by the experiments Calculated voltages of from -1 to -14 mV comprise only a small voltage range and the dependence of $D_{\lambda}^{KCl}/D^{KCl}$ on v could have been related in part to changes in membrane potential In the limits studied Eq 7 appeared valid within 10—20

Eq 3 which gave correctly the non-steady state flux of a single univalent salt could be reduced to Eq 4 for a thin membrane to give C_2 as a function of time For two univalent salts the exact formulation of the net flux ratio demands precise knowledge of the membrane concentration profiles The validity of the assumptions leading to Eq 8 is supported because the equation predicted correct net flux ratios when $S = 1$

Assumptions and Errors

The flux equation (Eq 1) neglects specific membrane-ion interactions as well as non-electrical cross effects among ions of different species (Rapoport 1964) Eqs 5 6 and 7 which were derived by integration of Eq 1 across the membrane are exact only if the membrane has homogeneous pores Sollner (1945) pointed out that collodion membranes are heterogeneous,

with probable electrical short circuits within them when they separate solutions at different concentrations It is also likely that the steady state ionic ratio depends on the distribution of pore radii since under a fixed pressure gradient the water velocity in each pore should vary as the fourth power of the radius Eqs 5 6 and 7 are probably approximations of a sum of micro scopic systems only a portion of which is in a microscopic steady state with zero electric current

The variances of the experimental results may have been due to changes during a run in D_o^{KCl} (changes in the order of 5 $_o$) or in other constants

Even with efficient stirring a membrane surface film may be 30 μ thick (Schulman and Teorell 1938) The film could possibly change under different flow conditions so as to produce a consistent error in the differences between zero fow and derived diffusion coefficients

As noted an underestimation of f^L and thus of Li^+ mobility within the membrane could explain variations in KCl diffusion coefficients Mobilities were calculated from limiting con ductance ratios which may be incorrect in significantly concentrated solutions but it would have been unwarranted to obtain mobilities from non limiting conductance ratios (Robinson and Stokes 1959 p 290) Dilution of membrane contents due to large water flows or to low initial conc ntrations in Compartment 1 might affect conductance quotients and thus mobili ties because of membrane charge

Thermodynamics of System

The experimental system with water flow is an open system of the first order since only the generalized force acting on water is held constant (DeGroot 1952 p 199) It may be treated in terms of irreversible thermodynamics (Nims and Thurber 1961 Rapoport 1964) Some first order open systems have been proposed as living cell analogies because they lead to ionic accumulation for the dependent (passive) ions and have characteristics of what has been called act ve transport (reviewed by Rapoport 1964)

In these experiments ΔP may be considered to be held constant by the transfer of water at the rate of *v* ml/sec from Compartment 1 at P to Compartment 2 at the higher pressure P_2 Work done *on* the system at the steady state compensates for the work dissipated *by* the system as water flows downhill from P to P carrying K^+ Li^+ and Cl^- against their respective con centration gradients in a work or energy coupled process

Thus the experiments demonstrate that accumulation (or ionic separation) can take place by coupl ng of two work terms or of two active mechanisms which need not be ion specific Eq 7 shows that in the presence of water flow accumulation occurs because of differences in the membrane mobilities of individual dependent ions

The accumulat on ratio increases when ionic concentrations in Compartment 2 decrease (cf Eqs 5 6 and Table I) If a living cell were to accumulate ions on the basis of water flo a second independent constraint would be necessary to produce equal total concentrations on both sides of the cell membrane

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A Micromethod for the Determination of Carbon Monoxide in Blood

By

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Abstract

Linderholm H *A micromethod for the determination of carbon monoxide in blood* Acta physiol scand 1965 64 372—376 — A method is described which makes it possible to determine small amounts of CO in gas and blood by the use of palladium molybdenum test tubes for the detection of CO 0 05 ml of blood with normal COHb saturation (about 0 5 per cent) corresponding to a total amount of about 0 05 μl CO is sufficient for an analysis of the CO content.

For the determination of the carbon monoxide content of the COHb saturation of blood in normal subjects (COHb saturation about 0 5 per cent) earlier described methods require comparatively large amounts of blood (Roughton and Root 1945 Sjösten and Sjostrand 1951 Lawther and Apthorp 1955 Gaensler *et al* 1957 Coburn *et al* 1964) The micro-diffusion method described by Berka (1955) based on the reaction between CO and palladium chloride requires only 0 2 ml blood for an analysis of the CO content but its error is not stated

In this paper a modification of the colorimetric method using a mixture of palladium and molybdenum compounds in indicator tubes (Shepard 1947 Andersson and Dahlström 1958 Dahlström 1960) will be described which allows determination of the CO content in as little as 0 05 ml of normal blood corresponding to a total amount of about 0 05 μl CO The method also allows the determination of small amounts of CO in gas

Method

Apparatus for extraction of CO from the blood

CO is extracted from the blood in a modified van Slyke apparatus described by Linderholm *et al* (1957 1965) see Fig 1 The extraction chamber has a volume of 100 ml and contains a magnetic stirrer Blood and reagents are admitted by the cup and through the 3 way stop-cock Extracted gas may be ejected through the stop-cocks to another gas recipient by means of

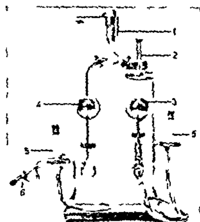


Fig. 1 The modified van Slyke apparatus 1 U tube 2 Cup 3 Extraction chamber 4 Gas recipient, 5 Leveling bulb 6 Needle valve

leveling mercury bulbs or to a wide U tube through which gas flows to the CO analyzer There is a needle valve in the rubber tubing from the recipient to its leveling bulb

Reagents Octylic alcohol Triton X 100 distilled water and sulphuric acid 10 per cent

Procedure

The van Slyke pipette including the capillary of the cup is filled with mercury Octylic alcohol and Triton X 100 0 5 ml of each and 4 ml of distilled water are added to the cup Blood (0 05—0 1 ml) is added by means of a constriction pipette made of glass or polyethylene (Mattenheimer 1961) Before blood is sucked into the constriction pipette some water is placed above the constriction After the blood has been sucked into the constriction pipette its upper end is connected to a rubber tubing containing CO free N_2 gas which is slowly flowing from a N_2 cylinder via a T tube open to the air The glass constriction pipette with a rubber tip is placed in the cup In case a polyethylene pipette is used it is first placed in a glass tube which is a little shorter than the pipette and with one end formed to a tip The hole of the tip is occluded by the tip of the polyethylene pipette when it is pushed 1—2 mm through the hole The glass tube has a rubber tip which is placed in the cup The blood sample is sucked into the extraction chamber followed by the water which rinses the construction pipette from blood and 10 ml of CO free N_2 gas. Afterwards about 4 ml of fluid in the cup are sucked into the extraction chamber and a mercury lock applied. The mercury surface is lowered halfway down in the extraction chamber to allow stirring After 2 min of stirring 1 ml of sulphuric acid is sucked into the extraction chamber and a new mercury lock is applied By lowering the leveling bulb the extraction chamber is almost emptied of mercury and the fluid is stirred for 7 min By means of the stopcocks and the leveling bulbs the gas in the extraction chamber is conveyed to the gas recipient After the addition of another 10 ml of N_2 gas to the extraction chamber and 3 min stirring under low pressure this gas volume containing the last traces of CO about 2 per cent of the total amount is conveyed to the gas recipient The gas collected in the recipient is then analyzed for its content of CO

CO-analysis

The CO is analyzed by ejecting the 20 ml gas column in the recipient at a fairly even rate for 40 sec into a O_2 gas stream which is sucked through the U tube of the modified van Slyke pipett and through the palladium molybdenum test tube[1] at an even rate of 50 ml min The ejection of the gas at a fairly even rate is obtained by placing the mercury leveling bulb of the gas recipient above the recipient so that the difference between the mercury surfaces is about 75 cm at the beginning and about 1 m at the end of the ejection. By means of a 3 way stopcock the mercury is passed through the properly adjusted needle valve The test tube is placed

[1] manufactured by LKB Stockholm Sweden

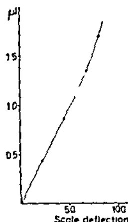


Fig 2 Relationship between units of scale deflection on the potentiometer writer (abscissa) and the amount of CO µl in the gas sample (ordinate)

in a Beckman B photometer modified as described by Andersson and Dahlstrom (1958) The colour change of the test tube occurring when CO is present in the gas stream changes the extinction for light of wave length 650 µm This is recorded on a potentiometer writer connected to the output of the photometer The deflection of the potentiometer writer 1 min after the deflection starts is within certain limits proportional to the amount of CO contained in the 20 ml gas sample of the recipient It should be noted that mercury vapour present in the gas produces a coloured ring in the first part of the palladium molybdenum indicator column This ring is however clearly distinguishable from the evenly distributed colour change caused by CO It is important to place the indicator tube in the photometer so that the mercury ring comes outside the light path

Calibration is made by adding known amounts of CO to the modified van Slyke pipette from a CO air mixture in a cylinder by means of calibrated syringes The gas volume in the syringes is injected into a slow stream of N gas which is sucked through a rubber tubing and a glass tube with a rubber tip placed in the cup into the extraction chamber up to a volume of 20 ml After transferring the gas from the extraction chamber to the gas recipient the gas mixture is conveyed to the indicator tube in the same way as when gas extracted from blood samples is analyzed

Comparison of deflections on the potentiometer writer for standard gas and for gas extracted from blood gives after correction to STPD the CO content of the blood sample The relationship between the deflection of the potentiometer writer and the amount of CO in the gas of the recipient is shown by Fig 2 Up to an amount of about 1 µl CO the relationship is linear but it is at a fairly constant temperature of 20—22 C influenced by the barometric pressure Calibrations close to the blood analysis are therefore recommended

COHb saturation The COHb saturation of the blood was calculated from the measured CO content in ml CO per 100 ml blood and the CO capacity of the blood which was obtained by multiplying the hemoglobin content in g per 100 ml of the sample by 1 34 The hemoglobin concentration was measured as oxyhemoglobin (Sunderman *et al* 1953)

Results

When various volumes of the same blood sample were analyzed the CO content was directly proportional to the volume (Fig 3) The standard error of a single determination was calculated from 10 —15 double determinations in different series performed by various technical assistants The standard error was 0 005—0 006 ml CO/100 ml blood at a mean CO content of about 0 1 ml CO 100 ml blood (range 0 063—0 107) or 0 02—0 03 per cent COHb saturation at a mean COHb saturation of about 0 5 per

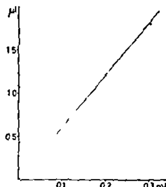


Fig 3 Relationship between the volume of blood ml (abscissa) and its CO content μl STPD (ordinate) The dots represent means of 3 determinations

cent (range 0 45—0 74) The error was about the same if constriction pipettes of 0 05 or 0 1 ml were used but it increased in proportion to the CO content of the blood sample In a series of 10 double determinations on blood with an average COHb saturation of 4 per cent and a mean CO content of 0 8 ml/100 ml blood the standard error was 0 036 ml CO/100 ml blood and the coefficient of variation was 4 5 per cent The coefficient of variation was thus rather constant independent of the CO content of the blood in the range that has been examined It varied in the different series between 3 5 and 6 per cent The error for the determination of CO in gas was smaller the coefficient of variation being 2 5—3 per cent

Comparison of the results of blood analysis with the present method and those obtained by analysis of 1—2 ml blood samples with the method described by Linderholm *et al* (1957 and 1965) agreed within the error of the methods

Discussion

The main advantage of the method is that only a small amount of human blood is required for a determination of its CO content Finger or earlobe blood can be used This facilitates measurements in situations when the rebreathing method (Sjostrand 1948) is difficult to apply for measurement of the COHb saturation for instance for studies on hemolytic conditions in new born infants Measurement of the diffusing capacity of the lungs with the steady state method with due regard to the back pressure of CO from the blood (Linderholm 1957) can now also be done with only finger or earlobe blood another necessary method for this purpose being already available i e a micromethod for the determination of arterial carbon dioxide tension from analysis of earlobe or finger prick blood (Maas and Heyst 1961 Koch 1965)

The error of the method is small for blood with a low CO content smaller than that of several other methods (Roughton and Root 1945 Gaensler *et al* 1957) A smaller error (2—3 per cent) may be obtained with the method described by Linderholm *et al* (1957) which however requires a larger amount of blood At concentrations of CO in the blood higher than that corresponding to a COHb saturation of 3—4 per cent the error of the present method increases above that given for several other methods

The greater specificity of the palladium molybdenum test tubes for CO than that of infrared meters or hopcalite apparatuses in several situations for instance when anesthesia gases are present is sometimes valuable (Linderholm and Lundstrom 1965)

Further advantages of the method are that the cleaning of the modified van Slyke apparatus is easy when these small amounts of blood are used that the analysis can be done with an equipment which with smal modifications is available at most laboratories and that it can be used for analysis of small quantities of CO in small gas volumes

Some disadvantages of the method are that the handling of the constriction micropipettes requires some training and that it is difficult to use for analysis of blood samples with a high CO content However for analysis of the CO capacity of blood the method described by Dahlstrom (1960) may be used as a complement.

Reasons for the use of N_2 gas in the extraction chamber and of sulphuric acid were discussed by Linderholm *et al* (1965) Is should be mentioned however that with the present method identical results were obtained either 1 ml of a 20 per cent potassiumferricyanide solution or sulphuric acid was used for the release of CO from blood

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Acta physiol scand 1965 64 377—382

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Direct Demonstration of the Systems of Terminals Belonging to an Individual Adrenergic Neuron and Their Distribution in Rat Iris

By

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Received 29 November 1964

Abstract

Malmfors T and Ch Sachs *Direct demonstration of the systems of terminals belonging to an individual adrenergic neuron and their distribution in rat iris* Acta physiol scand 1965 64 377—382 — The adrenergic nerves remaining in the rat iris following the destruction of most nerve cells in the superior cervical ganglion were studied with the use of a histochemical fluorescence method — The iris technique used have for the first time made it possible directly to demonstrate how the postganglionic adrenergic fibre by branching within the innervated tissue forms a system of long preterminal axons which run to different parts of the iris and how each preterminal axon finally forms a system of long and branching terminals (see Fig 5) The systems of terminals that belong to an individual neuron are distributed to areas spread over a large part of the iris The findings give direct evidence for the view of Hillarp (1946 1959) as to the construction of the autonomic innervation apparatus — The study also shows that one and the same neuron can give excitatory and inhibitory innervation respectively to two antagonistic effectors (dilator and sphincter)

Observations on the innervation of smooth muscles have shown that local contractions are usually not obtained even if only a few nerve fibres to the tissue are stimulated (Langley 1904) This shows that the postganglionic fibre must ramify extensively and innervate cells that are far apart from each other Studies on axon reflexes in the skin support this view (Lewis and Marvin 1927 Wagenaar 1931 and Wilkins *et al* 1938) Owing to the complex structure of the autonomic innervation apparatus (and the technical difficulties inherent in the neurohistological methods) very little is known however as to how this is brought about It has proved impossible to obtain any exact or more detailed knowledge of such fundamental problems as the extension distribution and general appearance of the terminals belonging to an individual postganglionic neuron The concept of a nerve ending as used in studies on autonomic effector systems, accordingly still lacks the necessary morphological basis

The adrenergic nerves and the adrenergic innervation apparatus can now be studied in an entirely new manner thanks to the highly sensitive fluorescence method of Falck and Hillarp which permits the direct demonstration of the adrenergic transmitter (see Malmfors 1964 1965a Norberg and Hamberger 1964) The distribution and construction of the terminal parts of the adrenergic nerves can be studied especially successfully and conveniently in whole mounts of albino rat iris which makes possible a close examination of the adrenergic axons and terminals in their entire extension throughout the tissue

The adrenergic nerves in the rat iris lose their noradrenaline (NA) content within 24 to 36 hrs after cervical sympathectomy and can consequently no longer be visualized with the fluorescence method (Malmfors and Sachs 1965) It was found however that one or two postganglionic neurons were sometimes left intact making it possible to examine the systems of branching terminals and non terminal axons arising from a single postganglionic fibre In the present paper these systems have been studied in detail with the use of irises in which nearly all the adrenergic nerves had degenerated following the destruction of most of the nerve cells in the superior cervical ganglion

Material and methods

About 100 female albino rats Sprague Dawley weight approximately 200 g were used In all animals the superior cervical ganglia were detached from the surrounding tissue and crushed with tweezers in such a way as to destroy most of the nerve cells Some animals were treated with nialamide (Niamid Pfizer 100 to 500 mg/kg i p 4 hrs before death) and NA (1 mg/kg i p 1 hr before death) This results in a marked increase in the NA content and consequently in the fluorescence intensity of the non terminal axons (see Malmfors 1965a) making them easier to follow

The animals were killed by decapitation under light ether anaesthesia 36 to 72 hrs after the operation The eyes were removed immediately and the irises prepared as whole mounts which after drying were treated with formaldehyde gas of optimum humidity (see Hamberger *et al* 1965) at + 80 C for 1 hr and then examined and photographed in a fluorescence microscope with an oil immersion condensor (for details see Malmfors 1965a)

Results

Thanks to the presence of NA throughout the neuron and to the high sensitivity of the method (see Norberg and Hamberger 1964) the adrenergic axons exhibit along their entire length a more or less intense green to yellow green fluorescence from NA converted by the histochemical treatment to a 3 4-dihydroisoquinoline (Falck *et al* 1962 Corrodi and Hillarp 1963 1964) The intact innervation of rat iris has been described elsewhere (Malmfors 1965a and b)

Following the destruction of a greater or lesser proportion of the nerve cells in the superior cervical ganglia the irises were found to contain a varying number of intact adrenergic nerves About one tenth of the irises showed such marked denervation as to be suitable for the purpose of this study In some of these irises the plexus of adrenergic nerves was strongly reduced throughout the iris or in more or less randomly distributed areas leaving just *one or two systems of branching axons arising from a single postganglionic fibre* (Fig 1) In other irises the fluorescent nerves left were so few that it was possible to follow a single fibre from its entrance into the iris at the ciliary body to the end of its outermost ramifications Since the non terminal axons — like the terminals —

F g 1 Most of the adrenerg c nerves have degenerated leav ng some pre e minal axons (→) and about fou systems of stronger fluorescent branching terminals × 160

F A system of b nching terminals arising from a single preterminal axon (→) is seen over the dilator muscle 160

Fig 3 A s stem of branching terminals arising from a preterminal axon is seen around an ar teriole × 160

Fig 4 A stem f b ti term nals in the sphincter zone and another over the dilator In the fluorescence m scope the two systems could clearly be seen to arise from the same main axon × 160

have an efficient mechanism for the uptake and concentration of NA (Hamberger *et al* 1964) they could be easily observed following the administration of this amine

A compilation of the findings made in these 20 or so irises reveals the following general pattern of distribution and appearance for the individual adrenergic axons in albino rat iris (Fig 5)

The postganglionic fibre arrives at the periphery of the iris at the ciliary body in a small nerve trunk usually perpendicular to the ciliary body Such a fibre will be referred to until the point at which it branches as the main axon The fluorescence intensity of the main axon is in untreated animals fairly low and uniform The main axon is about 0 7 to 1 μ in diameter and it branches at varying distances from the ciliary body Within a short space the fibre ramifies into several (about 4 to 6) thin (about 0.5 μ) branches These branches which are also weakly and uniformly fluorescent run in all directions to different parts of the iris They themselves do not seem to ramify to any major extent but they shortly change their smooth appearance slightly showing small enlargements like the varicosities of the terminals (see below) only smaller more irregular and of a lower fluorescence intensity These fibres will be referred to as the preterminal axons

Each preterminal axon ends with system of branching fibres of highly characteristic appearance (Fig 2) These latter show abundant more or less oval enlargements or varicosities (about 20 per 100 μ average thickness about 1 μ) which exhibit a strong fluorescence due to the presence of very high concentrations of NA The thin segments (about 0 2 to 0 5 μ) between the varicosities on the other hand exhibit a weak fluorescence giving these branching fibres — which will be referred to as the terminals — the appearance of a string of pearls

The extension of these terminal systems varied but those distributed to the dilator covered on the average an area of about 200 × 500 μ The terminals of a single system had a total length of several mm (3 to 4 mm in Fig 2) The terminals often ended with an enlargement quite similar to the varicosities but a number ended without any visible swelling

Not all the systems of terminals were distributed to the dilator muscle Some of the systems found were confined entirely to the sphincter region (Fig 4) The terminals in these ran mainly in the same direction as the muscle cells of the sphincter Some of the systems observed were localized in the walls of arterioles that were surrounded by the terminals (Fig 3) It was sometimes found that one of the preterminal axons gave rise to a system of terminals distributed to the dilator while another belonging to the same main axon ended with terminals in the sphincter region (Fig 4) or around an arteriole

In some cases only one postganglionic fibre arrived at the iris to give rise to preterminal axons and systems of branching terminals as described above This indicates that the adrenergic fibres travelling to the iris do not branch before their entrance in the target tissue always assuming that all the terminals of an individual neuron go to one the same effector organ

Discussion

Recent studies on the adrenergic innervation apparatus that have been made in this laboratory with the fluorescence method of Falck and Hillarp have furnished almost conclusive evidence for the view (Hillarp 1946 1959) that the fine terminal axon ramifications characterized by abundant varicosities are true terminals which store and

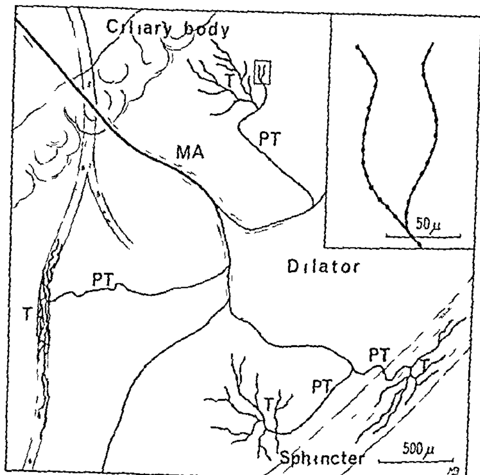


Fig 5 A schematic picture of how the axon of an individual adrenergic neuron extends throughout the iris The drawing is made up of pieces from different preparations In the upper left hand corner there is seen apart from an arteriole a single main axon (MA) which passes the ciliary body from the choroid in a small nerve bundle It then branches into thin fibres extending over a large part of the iris These thin branches the preterminal axons (PT) later change their appearance showing small irregular enlargements The preterminal axons finally split up into a system of branching fibres the terminals (T) These have a characteristic appearance with abundant more or less oval enlargements or varicosities (*Cf* the enlarged drawing of branching terminals in the upper right hand corner) Systems of branching terminals from one and the same main axon can be found in widely different parts of the dilator muscle in the sphincter region and around an arteriol

release the transmitter (see Norberg and Hamberger 1964 Malmfors 1965a Malmfors and Sachs 1965) Practically all the transmitter is stored in the varicosities which show very high concentrations of NA probably in the order of 10 000 μg/g wet wt see Norberg and Hamberger 1964 The observations made give also strong support to the view that it is these varicosities that are the presynaptic structures specialized for the

synthesis storage and release of the transmitter This could in fact be directly demonstrated by studying the disappearance of the stored NA on sympathetic stimulation in combination with inhibition of the transmitter resynthesis (Malmfors 1964)

The iris technique used in the present work has for the first time made it possible directly to demonstrate how a postganglionic adrenergic fibre, by branching within the innervated tissue forms a system of long preterminal axons which run to different parts of the iris and how each preterminal axon finally forms a system of branching terminals covering a fairly large area (see Fig 5) It follows that the systems of terminals belonging to an individual adrenergic neuron are distributed to areas spread over a large part of the iris This explains why local contractions of the dilator muscle are usually not obtained even if only a few postganglionic fibres are stimulated (Langley 1904)

The adrenergic innervation apparatus is constructed along the same lines also in several other tissues (Norberg and Hamberger 1964 Malmfors 1965b and c) It therefore seems probable that our findings as to the general appearance and distribution of the systems of terminals belonging to an individual neuron are in principle of quite wide application

Strong evidence has been obtained for the view (Hillarp 1946 1959) that the autonomic ground plexus — a two or three dimensional network each strand of which usually contains two or more fine and varicose axons — is the real innervation structure that it is built by long nerve terminals and that terminals from two or more neurons converge to and innervate the same group of effector cells (see Norberg and Hamberger 1964) The present findings give the first direct evidence for this view The adrenergic innervation apparatus of the iris dilator muscle in the rat appears as a dense two dimensional network in which two or more terminals usually run together (see microphotos in Malmfors 1965a) On the basis of the findings made in the present work it can be concluded that this network is made up of a large number of systems of long and branching terminals and that the terminals running together in the same strand originate from different neurons showing the correctness of the convergence principle Our findings incidentally disprove entirely the syncytical nerve net hypothesis (for references see Hillarp 1960)

The true endings of the terminals are quantatively insignificant and it seems unlikely that they can play any essential role in adrenergic transmission They may however be identical with some of the structures described by electron microscopists as true nerve endings which are intimately associated with — *e g* partly embedded in a pocket of — effector cells

The sphincter of the iris in various mammalian species shows an adrenergic innervation of varying abundance which in all probability has an inhibitory function (Malmfors 1965b) It is therefore of considerable interest that neurons giving terminals to the dilator were found also to supply terminals to the sphincter One and the same neuron can thus provide excitatory and inhibitory innervation respectively to two antogonistic effectors

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Acta physiol scand 1965 64 383—386

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The Metabolism of Fatty Acids in the Rat

VIII Lauric Acid and Myristic Acid

By

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Received 3 December 1964

Abstract

Goransson G *The metabolism of fatty acids in the rat VIII Lauric acid and myristic acid* Acta physiol scand 1965 *64* 383—386 — Either 1 C lauric acid or 1 C myristic acid together with 9 10 H palmitic acid were i v injected into fasted and refed male rats and the disappearance of label from the blood and the tissue distribution of label studied Both lauric and myristic acid disappeared more rapidly than palmitic acid from the blood In refed rats lauric acid was extracted even more rapidly than myristic acid Lauric acid was oxidized at a higher rate than myristic acid and myristic acid was more rapidly oxidized than palmitic acid More lauric acid label and myristic acid label was found in the liver Neutral lipid plus FFA than in the liver phospholipids This skewed distribution was most marked for lauric acid in the fasted rats The uneven distribution is discussed in relation to the fatty acid composition of the liver lipids to certain feeding experiments and to the interconversion with other fatty acids

In a series of articles the results from experiments in the rat with several fatty acids have been reported (Goransson and Olivecrona 1964 a and b Goransson 1964 a b c d and e) This paper deals with the findings in similar experiments where C lauric or C myristic acid was i v injected together with H^3 palmitic acid

Methods

The 1 C lauric acid (Batch 9 Spec act 21 0 mc/mM) the 1 C myristic acid (Batch 8 Spec act 15 4 mc/mM) and the 9 10-H palmitic acid (Batch 3 Spec act 389 mc/mM) were purchased from the Radiochemical Centre Amersham, England The H myristic acid (Spec act. 10 mc/mM) was tritiated from nonlabeled acid (The Hormel Institute Austin Minnesota USA) as described by Bergstrom and Lindstedt (1957) The acids were purified as described earlier (Goransson and Olivecrona 1964) prior to the preparation of the injection solution Half a ml of serum was injected into each rat This dose contained approximately 0 1 μeq of labeled lauric and 0 1 μeq of labeled palmitic acid or 0 1 μeq of labeled myristic and 0 1 μeq of labeled palmitic acid When C lauric and H myristic acid was injected the dose contained about 0 1 μeq of the former acid and 0 3 μeq of the latter acid

TABLE I Ratio C^{14}/H^{3} radioactivity in the blood neutral lipids plus free fatty acids in rats after the i v injection of C^{14} lauric acid or C^{14} myristic acid together with H^{3} palmitic acid in rat serum The ratio in the injected fatty acid mixture was taken as 1.0

	Lauric acid				Myristic acid			
Min	*Fasted rats*		*Refed rats*		*Fasted rats*		*Refed rats*	
1	0.8	0.7	0.4	0.4	0.9	0.8	0.6	0.6
2	0.8	0.7			—	0.8		
3	0.7	0.7			0.7	0.7		
4	—	0.7			0.7	0.8		
5	0.8	0.8			0.7	0.9		

TABLE II Ratio C^{14}/H^{3} radioactivity in tissue lipids from rats 5 min after the i v injection of C^{14} lauric acid or C^{14} myristic acid together with H^{3} palmitic acid in rat serum. The ratio in the injected fatty acid mixture was taken as 1.0 Total in the rat is the mean ± SEM of 5 rats

	Liver Total	Neutral fat plus FFA	Phospholipids	Adipose tissue Total	Carcass Total	Total in the rat
Lauric acid						
Fasted	0.23	0.37	0.04	0.23	0.47	0.31 ± 0.01
Refed	0.81	1.00	0.54	0.58	0.56	0.72 ± 0.02
Myristic acid						
Fasted	0.24	0.28	0.19	0.80	0.75	0.55 ± 0.01
Refed	0.90	1.07	0.67	0.91	0.92	0.90 ± 0.02

The nutritional state of the rats the operative procedure and the analytical methods were the same as those described by Göransson and Olivecrona (1964a) except for one modification In preliminary experiments it was found that myristic and specially lauric acid when present as free fatty acids were lost to a considerable degree when the lipid samples were taken to dryness using heat and a stream of nitrogen To avoid this loss of free myristic and lauric acid the solvents were evaporated simply by allowing the lipid samples to be assayed for radioactivity to stand at room temperature until dry The free fatty acid fraction was not separated from the neutral lipids in the present experiments

Results

As the values obtained for palmitic acid in this work agreed excellently with those published earlier (Göransson and Olivecrona 1964 a) the results in this paper are given as the ratio C^{14}/H^{3} in the individual samples The ratio in the injected fatty acid mixture was taken as one

The results of the present experiments can be seen in Table I and II. Table I shows that both myristic and lauric acid disappeared more rapidly from the blood than palmitic acid in both fasted and refed rats. The difference was greater for lauric acid than for myristic acid during the first min in the refed rats. This finding was verified in a separate experiment in which C^{14} lauric and H^3 myristic acid were injected to three rats. The ratio C^{14}/H^3 in the total blood lipids was then 0.55 ± 0.02 after 1 min and 0.31 ± 0.04 after 3 min.

Table II gives the tissue distribution of label 5 min after the injection. From the column "Total in the rat" it can be seen that the lauric acid was oxidized more rapidly than the myristic acid and that both fatty acids were oxidized more rapidly than the palmitic acid. Even in the refed rats a considerable amount of lauric acid was oxidized.

In the livers from both fasted and refed rats more C^{14} radioactivity was recovered from the "Neutral lipid plus FFA" fraction than from the phospholipid fraction both in the experiments with lauric and with myristic acid. This skewed distribution was most pronounced after the injection of lauric acid.

Discussion

Labeled free fatty acids have been shown to disappear from the blood at different rates (Goransson and Olivecrona 1964 b, Goransson 1964 a, b, c, d and e, Ono and Fredrickson 1964). In general the fatty acids seem to disappear more rapidly with increasing de saturation and with decreasing chain length. The last finding was further substantiated by the present experiments in which myristic and lauric acid were extracted faster than palmitic acid. In the refed rats the lauric acid disappeared from the blood even faster than the myristic acid. A possible dependence of the disappearance rate on the extent of association with serum proteins of free fatty acids was discussed by Goransson (1964 f). It was suggested that if the rate limiting step in the removal of fatty acids from the blood is the passage through a water phase, the solubility of each fatty acid in water should affect the disappearance rate.

In the present experiments carbohydrate refeeding did not reduce the oxidation of lauric acid to the same extent as the oxidation of palmitic acid. This finding supports the results obtained by Lossow and Chaikoff (1955) who established that the degree of sparing increases with increasing length of the carbon chain of the fatty acid. Lossow and Chaikoff studied the conversion of fatty acid C^{14} to expired $C^{14}O_2$, whereas the present technic determines the oxidation of fatty acid to nonlipid soluble products. However, any difference in the rate of oxidation of individual fatty acids should arise during the breakdown from fatty acid to acetate, since the acetate molecules derived from different fatty acids probably mix completely before further oxidation, resynthesis etc. Furthermore, it has been shown earlier (Goransson and Olivecrona 1964 a) that the oxidation from fatty acid to acetate is very rapid during the first few min after the injection. Thus the ratio between the recovered lauric acid label and palmitic acid label in the present experiments should be approximately the same as the ratio between lauric acid label and palmitic acid label not recovered as $C^{14}O_2$ in the work of Lossow and Chaikoff. This was indeed found to be the case. Using their data the above ratio is 0.70 and 0.37 for fed and fasted rats respectively and these values agree closely with the results presented in this work.

If rats are not fed excessive amounts of lauric and myristic acid the liver triglycerides contain small amounts of myristic acid and very small amounts of lauric acid. wl

the phospholipids contain negligible amounts of both acids (Dobiásova Hahn and Koldovsky 1964) The present results are in accordance with this as it was found that lauric acid was not incorporated into the phospholipids in the fasted rats to any appreciable extent This piece of information also supports the results of Elovson (1964) who found lauric and myristic acid in the liver only in the triglycerides after feeding hydrogenated coconut oil to rats

The difference in distribution of label between the liver neutral lipid fraction and the liver phospholipids from fasted and refed rats after injection of lauric acid suggests a rather extensive interconversion in the refed rats The interconversion has been studied by Elovson (1964) at our institute He found that in the fasted rats virtually no interconversion took place In the livers of refed rats about half of the radioactivity in the neutral lipids and one fifth of the radioactivity in the phospholipids was in lauric acid after this acid had been injected After injection of myristic acid approximately three quarters of the radioactivity was in myristic acid in both the neutral lipids and the phospholipids in the liver Both acids were principally converted to palmitic and stearic acid These findings by Elovson should be born in mind when the results in Table II in this paper are interpreted

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Acta physiol scand 1965 64 387—391

From the Department of Physiological Chemistry University of Lund Sweden

The Incorporation of Fatty Acids into the Alpha and Beta Positions of Liver and Adipose Tissue Lipids of the Rat

By

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Abstract

Goransson G *The incorporation of fatty acids into the Alpha and Beta positions of liver and adipose tissue lipids of the rat* Acta physiol scand. 1965 *64* 387—391 —Labeled lauric myristic palmitic, stearic palmitoleic oleic and linoleic acids were intravenously injected into fasted, male rats and the esterification at the alpha and beta position of glycerol in liver and adipose tissue lipids studied The fatty acids were rapidly incorporated into both alpha and beta positions of liver and adipose tissue lipids Each fatty acid was incorporated into the alpha and beta position in proportion to the amount at which that fatty acid normally occurs in each position. Thus the unsaturated fatty acids were mainly esterified at the beta position and the saturated acids at the alpha position

There is a marked difference in fatty acid composition among individual organs within a certain animal species and also among the lipid fractions of a particular tissue e g triglycerides and phospholipids (Goransson and Olivecrona 1964) A specific distribution of fatty acids between the alpha and beta positions in glycerides and phospholipids has also been noted (Menzel and Olcott 1964 Hanahan *et al* 1963 In order to study the mechanisms by which a specific fatty acid pattern is maintained in a certain lipid fraction several fatty acids were injected intravenously into rats Göransson and Olivecrona 1964 and 1965 Goransson 1965 a b c d e and f It became evident that several mechanisms such as preferential uptake interconversion and oxidation operated to modify the composition of the free fatty acids before esterification However the results also suggested a marked specificity in the esterification process itself This mechanism was studied in more detail by Goransson (1964) The results prompted the present more extended investigation of the esterification of fatty acids in rat liver and adipose tissue

TABLE I Total radioactivity and positional distribution of radioactivity in liver lipids and adipose triglycerides 5 min after the i v injection of labeled fatty acids dissolved in rat serum. Each value was obtained from a single animal. Alpha/beta denotes the ratio of the specific radioactivity in an average alpha position divided by the specific activity in the beta position

	Liver phospholipids		Neutral lipids plus FFA		Adipose tissue triglycerides
	Percent of injected radioactivity	Alpha/Beta	Percent of injected radioactivity	Alpha/Beta	Alpha/Beta
Lauric acid	0 6	5 8	4 2	—	2 8
Myristic acid	3 7	11	4 1	2 1	1 8
Palmitic acid	6 9	15	11	2 6	1 6
Stearic acid	8 4	27	—	—	—
Palmitoleic acid	2 0	0 4	4 8	0 6	0 7
Oleic acid	3 2	0 7	7 7	0 2	0 4
Linoleic acid	6 3	0 2	15	0.2	0 1

Methods

A C^{14} labeled and an H^3 labeled fatty acid dissolved together in rat serum were injected into fasted rats and the incorporation of label into liver and adipose lipids was determined. The positional distribution of the labeled fatty acids in glycerides and phospholipids was then studied using pancreatic lipase and phosphatidase A

The following labeled fatty acids used were obtained from The Radiochemical Centre Amersham England 1-C^{14} lauric acid (Batch 9 Spec act. 21 0 mc/mM) 1-C^{14} myristic acid (Batch 8 Spec. act. 15 4 mc mM) 9 10-H palmitic acid (Batch 3 Spec act [illegible] mc mM) 1-C^{14}-stearic acid (Spec act approximately 10 mc/mM 9 10-H -oleic acid (Batch 2 Spec act [illegible]00 mc mM) and 1 C linoleic acid Batch 20 Spec act 24 7 mc/mM) H palmitoleic acid (Spec act. 7 mc mM was biosynthetically prepared from 9 10-H^3 palmitic acid as described earlier (Goransson 19[illegible] Each fatty acid was purified by reversed phase chromatography and liquid liquid partition or thin layer chromatography to assure that more than 99 5 of the radioactivity was present as the appropriate free fatty acid The linoleic acid was subjected to AgNO SO thin layer chromatography as described by Goransson (19[illegible]) and the cis-linoleic acid recovered and shown to contain less than 0 5 of radioactive impurities.

The preparation of the injection solution has been described earlier (Goransson and Olivecrona 1964 0 ml of serum was injected into each rat. This dose contained about 0 08 μeq of one H labeled and 0 08 μeq of one C labeled fatty acid The low specific activity of palmitoleic acid made it necessary to inject about 1 0 μeq to produce satisfactory specific activities in the tissue lipids

The treatment of the rats prior to the experiments and the operative procedures were as reported previously Goransson and Olivecrona 1964 In the present experiments each rat was sacrificed five min after injection The triglycerides in the epididymal fat pads and the neutral lipids plus free fatty acids and phospholipids in the liver were recovered using extraction and chromatographic procedures essentially as described by Goransson and Olivecrona (1964) One modification that was introduced was dipping of the thin layer plates in a 2.5 solution of acetic acid in ether prior to development. This pretreatment of the plates ensured good separation of the diglyceride fraction from the free fatty acid fraction.

The liver triglycerides were not isolated from the other neutral lipids and the free fatty acids Thus the values given in Table I can not be considered strictly representative for the liver triglycerides The figures presented should however constitute a good approximation as it was found earlier that the radioactivity in the rat liver neutral lipids 5 min after the i v injection of labeled palmitic acid is mainly associated with the tri and diglycerides (Goransson and Olivecrona 1964 a)

The triglycerides from the adipose tissue and the neutral lip d plus free fatty acid fraction from the liver were subjected to degradation with pork pancreatic l pase contained in lyophil ized 100 000 × g supernatant from a 1 3 homogenate of the f esh gland 32 μM of lipid was taken to dryness in a 10 ml ampoule using heat and a gentle stream of nitrogen 3 8 ml of phosphate buffer (pH 8 0 0 15 M) and 200 μl of a lipase so ution were added Thus amount of lipase was shown in an experiment with C glycerol labeled triolein to give about 5 of total hydrolysis The incubation was carried out in the sealed ampoules for 1 hr at 37 C The lip d mixture was then recovered by l quid liquid partition and fractionated into tri di and monoglycerides and free fatty acids us ng thin layer chromatography The individual l pid fractions were eluted from the silica gel with 50 ml of 20 methanol in ether Radioactivity was measured by liquid scintillation counting and the glycerol content determ ned by the method of Carlsson and Wadstrom (1959)

The specific activity in an average alpha position of the liver neutral lipids and the adipose tissue triglycerides was derived by using the following formula

$$\text{alpha} = \frac{3 \times \text{TG} - \text{beta}}{2}$$

where TG is the specific activity of the triglycerides and beta the specific activity of the monoglycerides obtained by degradation The ratio between the specific activity of an alpha position and the beta position was then used in Table I It is obvious that uniform distribution of a fatty acid would result in a ratio equal to one

The total phospholipids from the liver were treated with phosphat dase A from bee venom (Leo Halsingborg Sweden) A solution containing 40 mg of phospholip d was taken to dryness in glass stoppered test tubes and the phospholip d emuls fied in 0 3 ml of phosphate buffer (pH 7 0 0 15 M) Phosphatidase A and 10 ml of ether were added and the tubes vigorously shaken for half a m nute The incubation was carried out overn ght at room temperature The solvent was then evaporated the l pids d ssolved in chloroform and transferred to S O -columns The free fatty acids were eluted with chloroform and the rema ning lip ds w th methanol When the metanol eluate was assayed by thin layer chromatography using chlo oform m thanol water acet c acid 75 25 3 1 as develop ng system only lysocompounds were detected It was concluded that the degradation of the beta ester bonds was complete To establish whether any alpha hyd olysis has occurred the lysocompounds were subjected to another degradation Only insign ficant amounts of f ee fatty a ids were liberated dur ng this p ocedure This suggested that no appreciable hydrolysis of the alpha ester bond had occurred during the first degradat on The labeled fatty ac ds in the chloroform fract on were therefore regarded as quantitatively recovered beta fatty acids

The subjection of the total phospholipid fraction to degradation with phosphatidase A constituted an approximation It had been shown earl er however that 80—90 % of the injected palmitic acid label in the phospholipid fraction were in glycerophosphatides (Goransson and Olivecrona 1964) Therefore the incorporation of label into nonglycerophosphatides in the present experiments should not significantly affect the interpretation of the data

The ratio between the specific radioactivity in the alpha and the beta position of the phospholipids given in Table I was calculated in the following way the radioactivity in the fatty acids spl t off by phosphatidase A i e the beta fatty acids was subtracted

from the amount of radioactivity in the total phospholipids used in the degradation The difference was taken to represent the radioactivity in the alpha fatty acids and was divided by the radioactivity in the beta fatty acids

Results and discussion

In Table I the percent of the injected dose is indicated for the liver phospholipid fraction and neutral lipid plus free fatty acid fraction These values are in good agreement with those obtained in earlier experiments (Goransson and Olivecrona 1964 and 1965 Goransson 1965 b c e and f)

The ratios α/β indicate a specific incorporation of the saturated fatty acids into the alpha position of the three fractions studied This tendency was most pronounced in the phospholipids where the incorporation of saturated fatty acids into the alpha position increased with increasing chain length

The unsaturated fatty acids were distributed predominantly in the beta position This distribution specificity was most pronounced in the case of linoleic acid

Information concerning the fatty acid composition in the alpha and beta position of rat liver lecithin and triglycerides is available in the literature (Menzel and Olcott 1964 Hanahan *et al* 1963) The ratio between the percentage of each fatty acid in the alpha position and the same fatty acid in the beta position can thus be formed For some of the fatty acids most abundant in the glycerophosphatides this ratio has the following value for palmitic acid 15 stearic acid 55 oleic acid 0 5 and linoleic acid 0 2 If these values obtained for lecithin are compared with the ratios of specific activity for the total phospholipids in this work the agreement for all the fatty acids except stearic acid is obvious The stearic acid is however so unevenly distributed that both the ratio in the present work and the ratio in the work of Menzel and Olcot (1964) may be somewhat uncertain Thus the conclusion to be drwan is that under the conditions used in the present experiments fatty acids are incorporated into the alpha and beta positions of the liver glycerophosphatides in proportion to the amount of each fatty acid in that position

A comparison between the data in Table I and the results obtained by Hanahan *et al* leads to the same conclusion for the liver glycerides as for the phospholipids above The similar incorporation of fatty acids into the liver glycerides and into the adipose tissue triglycerides suggests that also in the adipose tissue each fatty acid may be esterified in proportion to the amount of that fatty acid in each position

From the results in Table I it is clear that a rapid incorporation of label occurred into both the alpha and the beta position of the lipid fractions studied Thus the incorporation of label into the liver phospholipids should take place either by an extensive exchange of both the alpha and the beta fatty acid or by total synthesis as described by Kennedy (1957) For liver and adipose tissue triglycerides de novo synthesis seemed to predominate over exchange or incorporation into preformed partial glycerides This agrees with the findings of Shapiro *et al* (1960) who concluded that de novo synthesis was the main pathway for incorporation of labeled fatty acids in adipose tissue incubated in vitro Contrasting results were however obtained by Anderson and Tove (1964) These authors observed a higher specific activity in the fatty acids in the alpha position than in the beta position for a series of fatty acids after incubation with adipose tissue Only in experiments with adipose tissue from fed rats with glucose added to the incubation medium was the ratio of specific activity in the alpha position to specific activity

in the beta position below one for linoleic and oleic acid As these authors point out the difference may be attributable to reduction of alpha glycerophosphate in their in vitro experiments as compared with those of Shapiro *et al* This might render de novo synthesis less important than exchange and incorporation into partial glycerides Similarly the difference between the present results and those of Anderson and Tove may be explained by a more extensive supply of alpha glycerophosphate in vivo even though fasted rats were used

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Acta physiol scand 1965 64 392—397

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Electrophysiological Investigation of the Gustatory Effect of Ethyl Alcohol

I The Summated Response of the Chorda Tympani in the Cat, Dog and Rat

By

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Received 4 December 1964

Abstract

Hellekant G *Electrophysiological investigation of the gustatory effect of ethyl alcohol I The summated response of the chorda tympani in the cat dog and rat* Acta physiol scand 1965 *64* 392—397 —The summated response of the chorda tympani nerve to alcohol is characterized by an initial depression a slow onset of the discharge and then a strong positive response to a water rinse The initial period of decreased activity is related to the alcohol concentration in such a way that a stronger solution diminishes the period of depressed activity in the nerve The increase of activity is greater in the cat and the dog than in the rat This positive response in the cat and rat shows a maximum after which no further increase in the response can be observed with increased alcohol concentration Evidence is presented that the strong response to water after alcohol emanates from gustatory fibres which respond positively to water The possibility of rat gustatory responses to water and the types of gustatory fibres responsive to alcohol are discussed

The gustatory effect of alcohol has been electrophysiologically investigated only in a few studies Beidler (1953) reported that the response to alcohol was hardly perceptible in the rat In a recent paper Diamant *et al* (1963) reported that they had tested the effect of alcohol on the tongue of 2 human subjects and 1 dog by recording the electrical response from the chorda tympani nerve This response differed in several respects from that observed to other taste stimuli Therefore it seemed of interest to study the response in more detail using a number of species

Methods

12 rats (Sprague Dawley 4 dogs and [illegible] cats were used in this study They were anesthetized with Mebumalnatrium 1 ml of which contains 18 mg pentobarbital 40 mg pentobarbitalsodium and 250 mg urethan The initial dose of 0 6 ml/kg b w was administered i p in all species and the repeated doses i v in the cat and the dog

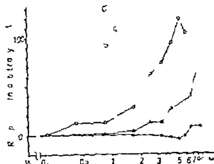


Fig 1 Curves of the summated response in the chorda tympani of the cat dog and rat to different alcohol solutions The response was measured in per cent of the response to Ringer s solution after 10 sec

The operative procedure and the experimental technique were almost the same as those used by Cohen Hagiwara and Zotterman (1955) The recordings were always obtained from the whole chorda tympani nerve

The gain of the summator was adjusted to give a good response to the application of the test solutions. These were applied to the tongue by means of a burette fixed rigidly in position with its tip a few mm above the tongue A two-way stop cock was connected to a tap water container and to the column of the burette The temperature of the water in the container was kept constant and equal to that of the bath in which the stimulus solutions were kept (29—33 C) The temperature of the tongue surface was recorded with a thermocouple The quantity (25 ml) and rate of flow (100 ml/min) of the test solutions were kept constant during the test. The tongue was rinsed with tap water after each test until the nerve showed its original activity Since no differences were observed between the responses obtained to solutions made up in distilled water or tap water tap water for practical reasons was used as a solvent

The tongue of the rat is small and it is difficult to expose its whole surface A catheter was therefore put in the esophagus and the solution is flowed from the posterior oral cavity out over the tongue

A two-bottle preference test with alcohol solutions and distilled water was carried out on the rats Each animal was kept in a separate cage The alcohol solutions in this experiment were made up in distilled water Two pipettes containing water or alcohol were inserted through holes in the lid of each cage The bottles were shifted once a day between and in the cages according to a random table (Fisher and Yates 1949) Thirteen solutions were used with concentrations between 0 1—? 5 M A statistical study was made on the results with Student s t test.



Fig ? The recording shows the summated response to 4 1 M alcohol in the chorda tympani of the cat The signal indicates the flow of alcohol Observe the initial decrease of the nervous activity Time marker 1 per sec

Results

In all the species tried the magnitude of the response to alcohol was moderate which can be seen in Fig 1 The figure shows the height of the summated response to alcohol plotted against the molar concentration The response was measured 10 sec after the application of the alcohol solution and given in per cent of the response to Ringer s solution The figure shows that a response was elicited with weaker alcohol solutions in the cat than in the dog and that the response was very small in the rat, even when strong solutions were used In the rat the summated response to alcohol in the chorda

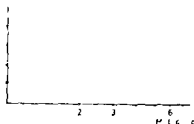


Fig 3 The reciprocal of the interval in sec between the onset of an alcohol flow and the moment when the summated activity in the nerve reached 2 mm above the level of spontaneous activity has been plotted on the ordinate and the logarithm of the molar concentration on the abscissa for one cat

tympani is small even measured in absolute terms Fig 5 demonstrates this clearly In the cat the response showed a maximum at 5 0 M alcohol and further increase in molarity only decreased the response In the rat a maximal response was obtained with the 6 5 M alcohol solution and still stronger solutions produced no change in the response

In the rat 1 6—4 9 M alcohol elicited a depression of the neural activity which lasted as long as the tongue was exposed to alcohol This depression can be seen in Fig 1 which also indicates that 4 9 M alcohol caused the greatest average depression in the rat This observation was confirmed by taking the mean levels for 10 sec periods before during and after the application of the alcohol in all animals The decrease in activity was more evident in some animals than in others Thus 2 rats out of 12 showed no decrease at all

An initial depression to alcohol was also observed in the cat (Fig 2) and in the dog (not illustrated) In these animals however the depression lasted only a few sec and then the summated response increased in magnitude This explains the lack of a depression in the curves from the cat and dog in Fig 1 as the values in this figure were obtained after 10 sec of alcohol flow when the depression was already over

Diamant *et al* (1963) suggested that alcohol depressed the activity of the receptors responding to water in the dog Further it has been shown by Cohen *et al* 1955 that in the cat salt depresses the activity of fibres sensitive to water Provided that the theory of Diamant *et al* is correct a combination of salt and alcohol would therefore be expected to cause a still stronger depression In a few experiments the effect of alcohol in Ringer s solution was therefore tested in the cat Unfortunately however the Ringer s solution elicited a salt response which masked the eventual depression If on the other hand the alcoholic Ringer was preceded with ordinary Ringer s solution the water response was already depressed Thus no direct evidence could be obtained about the type of fibres depressed by alcohol but a comparison between the initial decrease and the response to water rinse revealed that animals which showed a marked initial neural decrease to alcohol showed a strong response to a following water rinse

Fig 3 indicates that the duration of the depression shortened with increased concentration of alcohol The figure gives the results of a series of alcohol solutions of different concentrations applied to the tongue of a cat In the recording thus obtained the level of activity during a constant flow of water was used as a reference line The interval was measured between the moment when the solutions were applied and the point when the depression was altered to a definite increase in activity This was arbitrarily defined as a point 2 mm above the reference line The reciprocal of this interval was calculated and plotted on the ordinate in the figure The logarithm of the molar concentration of the alcohol solutions was plotted on the abscissa It should be mentioned that the

depression caused by alcohol varied in size from cat to cat and was sometimes practically absent (Fig 4 A)

The response to alcohol differed from the response to the conventional taste solutions in the respect that a water rinse following the exposure to alcohol caused a strong increase of activity This can be seen in Fig 4 A which shows the response to water after 3 3 M alcohol in the cat The signal indicates the duration of the flow of alcohol over the tongue A similar response to water after alcohol was seen in the dog and to a slight extent in the rat (Fig 5) Fig 4 B indicates that no similar response could be observed when the tongue of the same cat was rinsed with Ringer s solutions after 3 3 M alcohol in Ringer s solution

Fig 4 C and D shows two consecutive recordings obtained in one cat The response to distilled water after 4 9 M alcohol in distilled water is recorded in C. Fig 4 D shows the response to 0 5 M sucrose after 4 9 M alcohol in 0 5 M sucrose It should be observed that the alcohol solutions in Fig 4 B and D were made up in Ringer and 0 5 M sucrose respectively to prevent a response caused by the solvent The figure indicates that a rinse of 0 5 M sucrose in water after alcohol elicited a response similar to that caused by water after a previous alcohol application

Because of the relatively low response obtained from the chorda tympani of the rat, one may suppose that the threshold to alcohol is very high or the number of gustatory

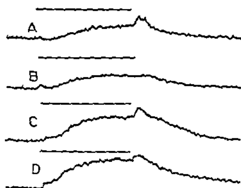


Fig 4 Recordings showing the summated response in the cat s chorda tympani nerve to
A 3 3 M alcohol in distilled water Distilled water before and after
B 3 3 M alcohol in Ringer s solution Ringer s solution before and after
Recordings A and B were obtained from the same cat Note the difference in activity when the alcohol was rinsed away
C 4 9 M alcohol in distilled water Distilled water before and after
D 4 9 M alcohol in 0 5 M sucrose 0 5 M sucrose before and after Recordings C and D are from the same cat and consecutive Observe the similarity in the increase of activity to the rinse of the sucrose solution and the distilled water after the alcohol In all recordings the horizontal bars indicate the flow of alcohol solutions Time marker 1 per sec



Fig 5 Recording of the summated response of the chorda tympani nerve in a rat to 7 4 M alcohol solution flowed over the tongue Distilled water before and after Observe the increase of activity when the solution was rinsed away Time marker 1 per sec

receptors sensitive to alcohol is rather small Further the stimulus of alcohol in the rat as well as in other species may be transmitted by fibres in other nerves The latter theory seems to be favoured by some experiments on lightly anesthetized rats It was observed that when 6 6 M and stronger alcohol solutions flowed over the tongue the recording showed artifacts due to swallowing movements although the recorded response to alcohol was weak Neither 0 5 M NaCl nor 0 5 M sucrose produced this effect although they elicited a much stronger response in the chorda tympani Injection of d tubocurarine abolished completely the artifacts and proved that they were due to muscular movements These experiments seem to indicate that the rat strongly experienced the alcohol stimulation in spite of the weak response in the chorda tympani (Fig 5) Similar reflexes were also observed in the cat In this animal however the muscular movements seemed to become most intense when the tongue was rinsed with water after alcohol in contrast to the rat which reacted strongest to the alcohol

A behavioural method was also applied to determine the threshold to alcohol in the rat A two-bottle preference test was employed with 5 rats The first sign of alcohol rejection was observed in the choice between 1 5 M alcohol and water ($p < 0\ 05$) and the aversion was evident at 2 2 M ($p < 0\ 005$ Student s t test two-tailed) This is interesting as Fig 1 indicates that the first depression of the neural activity was observed at 1 6 M alcohol

Discussion

The most striking characteristics of the response to alcohol in the chorda tympani were the initial depression the slow onset of the discharge and in the cat and the dog the strong response to a water rinse after alcohol exposure

In the rat the threshold to alcohol seemed to be high if judged by the increase of the summated discharge in the chorda tympani nerve The determination of the threshold with a two-bottle preference test gave a lower threshold to alcohol Fig 1 indicates that the first depression of the neural activity was observed at 1 6 M alcohol The first sign of an aversion to alcohol was observed at 1 5 M solution The observation on an aversion to this concentration is supported by Arvola and Forsander (1961) who reported an aversion to 10 % (1 8 M) alcohol in rats This may indicate that the neural depression caused by the alcohol was behaviourally significant as the threshold determined by the preference method was close to the alcohol concentration which first caused a decrease of the neural activity Neither the influence of olfaction nor the possibility of a sensory input via other nerves have been taken into consideration The threshold may seem high compared to a detection threshold in man of 0 5—0 7 M (Diamant *et al* 1963 Renqvist 1920) but it is lower than the values reported from some insects (Dethier and Chadwick 1947 Hodgson 1951)

The observation on lightly anesthetized rats revealed that the animals received information strong enough to elicit the reflexes even when the response in the chorda tympani was weak The observation may be explained in two ways One is that the response to strong alcohol may partly be due to an activation of thin fibres in the chorda tympani which is not disclosed by the summator The second explanation is that other nerves also mediate the response to alcohol

Diamant *et al* (1963 reported that alcohol produced a mild depression of the spontaneous nerve activity in the dog The present results are in agreement with this observation and in addition demonstrates a similar depression in the cat and the rat It may be of interest that the latency of the positive response to alcohol and the concen

tration of alcohol showed a similar relationship as the one found on quite different species with another technique (Cole and Allison 1930) These authors studied the relation between the alcohol concentration and the response latency in Balanus tintinabulum Rana pipiens and Planaria dorotocephala They found a linear relation between the reciprocal of the reaction time and the logarithm for the molar concentration

As previously mentioned Diamant *et al* (1963) proposed that the depressing effect of alcohol "was due to a depressing action on the receptors responding to water In the present investigation no direct evidence for this assumption was obtained but a correspondence between the strength of the initial depression caused by alcohol and the height of the response to water alcohol was observed The hypothesis will be further supported by the results of a following investigation

The response to water after alcohol may be explained if we presume that this response emanated from the water fibres described by Cohen *et al* (1955) It is then easy to predict that Ringer s solution after alcohol in Ringer s solution should not elicit a response as Ringer s solution does not stimulate the water fibres This explains the difference in the response in Fig 4 A and B to water and to Ringer s solution Liljestrand and Zotterman (1954) have shown that a response in the water fibres also can be elicited with a sucrose solution It has been observed in the present investigation that 0 5 M sucrose after alcohol in sucrose elicited a strong response in the cat as Fig 4 D shows These observations support the hypothesis that the response to water after alcohol is a response from water fibres

The response to water in the rat after the exposure to strong alcohol solutions and the observed initial decrease in the electrical response to an alcohol rinse indicate that even the rat may possess some ability to react to water by means of its gustatory mechanism although this ability is small and not as pronounced as in the cat and the dog Furthermore a positive response to water in the rat was reported by Pfaffmann and Bare (1950)

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Acta physiol scand 1965 64 398—406

From the Department of Physiology Veterinärhogskolan Stockholm Sweden

Electrophysiological Investigation of the Gustatory Effect of Ethyl Alcohol

II A Single Fibre Analysis in the Cat

by

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Received 4 December 1964

Abstract

Hellekant G *Electrophysiological investigation of the gustatory effect of ethyl alcohol II A single fibre analysis in the cat* Acta physiol scand 1965 64 398—406 — The response of single gustatory fibres of the chorda tympani in the cat to alcohol has been recorded and compared to the responses to NaCl sucrose acetic acid and quinine Alcohol evoked a response in all gustatory fibres tested In regard to their response pattern two principal groups of fibres have been observed In the first group alcohol produces an initial decrease of the spontaneous activity followed by a delayed and moderate increase When the tongue is rinsed with water after the alcohol there is a strong burst of impulses In the second group alcohol produces an immediate response characterized by a moderate increase in impulse frequency A subsequent application of water does not give rise to any burst of impulses in this group but brings the impulse frequency back to the resting level An analysis of the response pattern of single gustatory fibres revealed also that the first group of fibres are predominantly sensitive to water acid and quinine but is usually not stimulated by NaCl The second group is generally activated by salt

Studies in this laboratory (Diamant *et al* 1963 Hellekant 1965) have shown that the summated activity in the chorda tympani of the cat and the dog increases when the tongue is rinsed with water after exposure to alcohol This increase of activity was not or only to a slight extent observed when a rinse with Ringer's solution followed the alcohol (Hellekant 1965 Further it has been shown earlier (Liljestrand and Zotterman 1954) that the dog and the cat possess fibres sensitive to water It was suggested (Hellekant 1965) that the summated response to water after alcohol was due to an activation of fibres sensitive to water The activity of these fibres is depressed by salt It did therefore seem of interest to examine in detail the relationship between the fibres sensitive to water and the observed neural discharge to water after alcohol in an animal that possesses fibres which respond positively to water

An initial decrease of activity to alcohol in the chorda tympani was reported in a previous paper Hellekant 1965 It was proposed that the initial decrease and the

observed increase of sensitivity to water after alcohol might be related since they usually occurred together The present work was undertaken primarily in an attempt to analyze the relationship between the responses to water and alcohol and secondarily to compare the response to alcohol with the responses to the 4 conventional taste qualities in chorda tympani fibres

Methods

The cats were anesthetized with a 6 % solution of Mebumalnatrium 1 ml of which contains 18 mg pentobarbital 40 mg pentobarbitalsodium and 25 mg urethane The initial dose of 0 6 ml/kg b w was administered i p and the repeated doses i v The chorda tympani nerve was dissected according to the technique of Zotterman (Cohen, Hagiwara and Zotterman 1955) The epineural sheath was removed under a dissecting microscope The nerve was first divided with a sharp surgical blade into at least 8 bundles which then were split with fine needles into small bundles each containing a few or a single responsive fibre

The operative field was covered by a pool of Ringer s solution kept at body temperature For recording the nerve bundle was placed across a platinum electrode which was fixed in a micromanipulator Most of the Ringer s solution was drawn out and a second electrode was put in contact with the remaining solution The electrodes were connected to a balanced input RC coupled preamplifier and the amplified action potentials were then recorded on a cathode ray oscilloscope and a Tandberg stereotape recorder

The method of temperature control and application of solutions has been described earlier (Hellekant 1965) The technique permitted a constant adapting flow of water between the taste stimulations This kept the tongue at a constant temperature and free of saliva Irregularities in the flow when changing from water to a taste solution were also avoided

The solutions were made up in tap water which had a dry residue of 0 012—0 020 % ash 0 010—0 015 chloride 0.23 –0 28 mM magnesium 0 16—0 25 mM calcium 0 49—1 10 mM potassium and sodium together 7—8 mg/l and with a pH round 7 5 The reasons for using tap water will be discussed later 0 5 M sodium chloride 0 5 M sucrose 0 05 M acetic acid and 0 05 M quinine (monohydrochloride hydrate were used as test solutions The alcohol solutions were made with 95 % ethanol and tap water and had the concentrations 1 7 2 5 3.3 4 1 4 9 5 7 and 6 5 M Sometimes also a 8.2 M solution was tested

Two methods were used to construct Table I presenting the results on the fibre specificity One was based on a subjective determination of the number of qualities to which each fibre responded The second method was based on the assumption that the spontaneous irregular frequency can be described by a Poisson distributed time series A χ^2 test founded on the relation between λ and s was used to test this assumption In the cases the χ^2 lies inside the confidence limit of 0 05 this assumption was regarded as true Then the number of impulses during each of two consecutive seconds which exceeded the 99 confidence limit of a Poisson distribution was regarded as a response The 99 level was very arbitrarily chosen because of the availability of a suitable Poisson table in Documenta Geigy The differences in the results obtained with the two methods are rather small

Results

A total of 33 fibres from the chorda tympani of the cat were tested for their response to alcohol and other sapid solutions All of these gustatory fibres which responded to various sapid solutions such as NaCl acetic acid quinine hydrochloride and sucrose in the following called the standard solutions responded to alcohol Thirty per cent of the fibres tested responded with an increase in activity to a concentration of 1 7 M alcohol Ninety per cent responded to 2 5 M alcohol

The fibres were divided into two groups according to their response to alcohol and water after alcohol water fibres and non water fibres

Water fibres This group included 18 of 33 tested fibres Fig 1 A is a recording from a typical fibre of this group and shows several characteristics common to the group which will be described The signal on the recording represents a flow of alcohol over

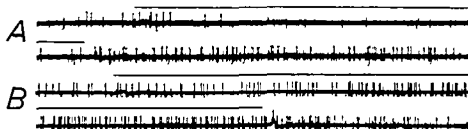


Fig 1 Response from two single chorda tympani units to a flow of 3 3 M alcohol which was preceded and followed by a constant flow of water The horizontal line indicates a flow of alcohol over the tongue Note in fibre A the initial depression to alcohol and the very strong response to water after Eight sec of the recording was excluded Note that fibre B did not show any initial depression to alcohol but a constant increase of the frequency which decreased immediately when the tongue was rinsed with water Time marker 10/sec

the tongue During the continuous water flow preceding the alcohol the discharge of the fibre recorded from was rather constant Some tenths of a second after the change from water to alcohol flow the discharge frequency decreased to about half of the value it had during the exposure to water This is evident from Fig 1 A showing the response to 3 3 M alcohol For practical reasons 8 sec of the recording was excluded in this figure The duration of the depression depended on the alcohol concentration used After the period of depression an increased impulse activity followed The recording in Fig 1 A indicates no increase during the alcohol flow but shows that when the alcohol was rinsed away by water the frequency in the nerve fibre was substantially increased This sudden burst of activity rapidly subsided After about 15 sec the fibre showed the same frequency as before the alcohol flow

Fig 2 gives a better demonstration of the moderate increase of the activity in two water fibres and the very intense response to water after alcohol The water fibres are represented by the circles and the dots The frequency per sec is plotted on the ordinate and the number of sec after the onset of a flow of 2 5 M alcohol over the tongue on the abscissa The line below the abscissa indicates the alcohol and the water flow

Some of the features which characterize and distinguish the water fibres from the other group are further demonstrated in Fig 3 (the triangles) In this figure only fibres tested to at least 3 consecutive alcohol concentrations were included The number of impulses during the 6th sec was plotted against the molar concentration Each triangle symbolizes the mean value of the activity in the number of fibres indicated by the figures beside the symbols The horizontal lines show the S E The curve obtained resembles very much the summated response from the whole of chorda tympani when plotted against molar alcohol concentration It is further evident from the figure that 5 sec after the onset of the flow of 1 6 M alcohol the frequency in the water fibres was lower than during the water flow Expressed numerically the difference is 19 and the reduction would have been more pronounced if measured after a shorter interval

In Fig 4 the frequency during the maximal response to water after each alcohol solution has been plotted against the logarithm for the concentration of the preceding alcohol solutions The curve shows a considerably more linear course for water than is shown in Fig 3 for alcohol

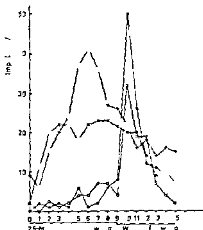


Fig 2 Curves of the responses to ? 5 M alcohol and water in four different units The frequency at time 0 sec was obtained during a constant flow of water The signal below the abscissa indicates when the flow was changed from alcohol to water

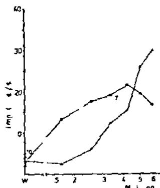


Fig 3 Shows the mean response to alcohol during the 6th sec in all fibres tested with at least 3 subsequent alcohol concentrations The triangles represent the mean value of the number of "water" fibres indicated by the figures beside the symbols The circles represent the mean value of the number of "non water" fibres indicated by the figures beside The oblique and horizontal lines show the S E for the two groups Note that the curve obtained from the water fibres shows a similar concavity as the one which was obtained when the summated response from the whole nerve was plotted to the concentrations of alcohol

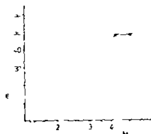


Fig 4 The mean value of the maximal frequency to water after alcohol in the same water fibres as in Fig 3 has been plotted against the logarithm for the concentrations of the preceding alcohol solution The horizontal lines indicate the S E The figures close to the points show the number of values in each point

Fig 5 shows further some of the features which characterize and distinguish water fibres from non water fibres In the first four columns of the figure the responses to the standard solutions are drawn as graphs Each column shows the difference between the activity during the first sec of stimulation and the spontaneous activity the last sec before In the 5th column the continuous line represents the maximal response to a flow of 4 1 M alcohol and the dashed line in the same column the

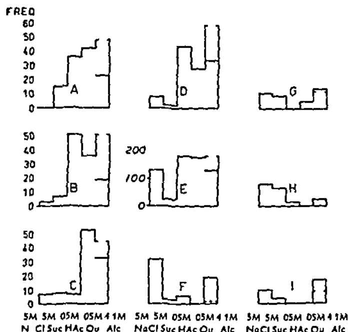


Fig 5 Histograms summarizing frequency of responses during the first sec to the standard solutions and the maximal impulse activity to 4 1 M alcohol in 8 different units Histogram E shows the relative magnitude of the summated response to the test solutions in arbitrary units The horizontal dotted line indicates the response to water during the first sec after the 4 1 M alcohol rinse Note the responses to water in A—D which were classified as "water" fibres and that the summated response in F briefly speaking is a sum of A—D and F—I

response to water during the first sec after the alcohol flow had ceased The spontaneous activity has been subtracted also from these values A—D in the figure were classified as "water" fibres The fibres in Fig 5 B and C are also presented in Fig 2 The summated response from the whole chorda tympani is added as a comparison in Fig 5 F It is evident from the figure that these fibres were stimulated very little or not at all by NaCl and sucrose In these fibres quinine or acid or both solutions stimulated to a very great extent The figure indicates that the positive response to water after alcohol in the "water" fibres usually exceeded or reached the same intensity as the response to the standard solution which stimulated the fibres most It shows further that the positive response to alcohol was moderate compared to the response to acetic acid or quinine or both solutions In 70 % of all "water" fibres the maximal response to alcohol was less than that of the standard solution which stimulated the fibre most The graphs show this clearly

Non water fibres In Fig 1 B the difference between this type of fibres and the "water" fibres can be observed The record demonstrates that the impulse frequency in the nerve fibres did not decrease when the animal s tongue was exposed to alcohol On the contrary the frequency slowly increased When the tongue was rinsed with water the frequency decreased to the same value as during the spontaneous activity without showing any increase of activity to the water flow

This difference between the two types of fibres is more apparent in Fig 2 where the responses of two of these "non water" fibres are indicated by crosses Further the figure

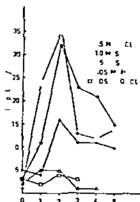


Fig 6 Curves of impulse frequency for NaCl sucrose acid and quinine in a "non water fibre Note the slow onset of the response to sucrose compared to that of sodium chloride and lack of response to acid and quinine The spontaneous activity during the flow of water is marked at 0 sec

shows that these fibres were stimulated to a greater extent by weak alcohol solutions than the "water' fibres

The last observation is more evident in Fig 3 where a number of non water fibres is plotted in the same way as for the "water fibres The circles represent the mean value of the number of impulses per sec The S E is represented by the oblique lines The figures beside the circles show the number of fibres included in each value It is evident from the figure that these fibres were stimulated to a greater extent by weak alcohol solutions The figure indicates that the curve rises approximately linearly between 1 6—4 1 M and falls at higher alcohol concentrations A similar decrease was observed in the summated recording from the whole chorda tympani in the cat (Hellekant 1965)

These fibres showed also other differences in comparison to the water fibres In Fig 5 F—I the responses from 4 typical non water fibres were marked similarly as the responses from the water fibres The fibre in Fig 5 G is the same as in Fig 1 B It is evident that these fibres were not stimulated by water after alcohol were stimulated weakly or not at all by quinine or acid to some extent by sucrose and strongly by NaCl The maximal response measured in impulses per sec when the tongue of the cat was rinsed with alcohol was usually greater than or equal to the response to the standard solution which stimulated the fibre in question most The recorded response to sucrose which can be seen in Fig 5 G—H is of interest Such observations were made on a total of 7 non water fibres Some of the non water fibres were therefore also tested with a series of sucrose solutions

Fig 6 chosen as an example shows the response to the 4 standard solutions and to 1 M sucrose The figure indicates that the frequency in the nerve increased when this strong sucrose solution was used It is also evident that the response to sugar did not show the same rapid increase as to NaCl and that this fibre was not stimulated by acid or quinine

Table I summarizes the observations made on the specificity to the standard solutions The figures within parenthesis in the table are based on the method described earlier It is obvious that there are borderline cases between "non water fibres which were stimulated by quinine and acid and water fibres which were stimulated by NaCl The majority of the two groups showed however the outlined specificity

TABLE I The distribution of fibres according to their responses to the 4 standard solutions used

Number of fibres responding to	Water fibres	Non water fibres
Sodium chloride alone	0 (0)	3 (3)
Acetic acid alone	1 (0)	1 (0)
Quinine hydrochloride alone	3 (3)	1 (1)
Sodium chloride and sucrose	0 (1)	5 (4)
Acetic acid and quinine hydrochloride	5 (5)	0 (0)
Sodium chloride acetic acid and quinine	2 (1)	1 (1)
Sucrose acetic acid and quinine	4 (4)	0 (0)
Sodium chloride sucrose acid and quinine	2 (2)	2 (2)
Only tested with sodium chloride and sucrose	1 (1)	
Not responding to any standard solution	0 (1)	2 (4)

Discussion

The results of this study show that the strong peripheral response to alcohol in the chorda tympani of the cat is built up of individual fibre responses which can be divided into two groups Further the investigation has shown that the taste fibres can be grouped according to their sensitivity to the conventional test solutions used and that there is a conformity between their response to alcohol and this sensitivity

Before the discussion of these observations something should be mentioned about the methods The main motive for using tap water instead of distilled water in the solutions was purely practical The procedure seemed to be justified by earlier experimental observations on the water response in the cat by Liljestrand and Zotterman (1954) who reported 'Tap water had nearly as strong an effect as distilled water Ringer's solution containing salts suppressed the response from the fibres sensitive to water therefore it could not be used as a solvent (Hellekant 1965)

Most of the fibres were exposed to a series of alcohol solutions and since the difference between the reaction pattern of the two groups is rather obvious the classification in water and non water fibres was not difficult In order to compare the fibres with earlier classifications of the fibre types they were grouped according to their sensitivity to the 4 taste qualities As the determination of the occurrence of a response in a fibre was subjective a further method for forming an estimate was used This method earlier described is the basis for the figures within parentheses in Table I A comparison of the results from the estimate based on observation and the statistical estimate showed in the main that the number of fibres which did not respond to any stimulus increased from 2 to 5 For the rest of the fibres the differences in the values obtained by the two methods were insignificant

The water fibres in this study seem to correspond to the fibres earlier classified as water fibres by Cohen *et al* 1955 These fibres were also sensitive to quinine and acid and not at all or weakly stimulated by NaCl This is in accordance with the characteristics of the water fibres in this study Sato (1963) reported a similar relation in the cat between the water sensitivity and the lack of a response to salt His water sensitive units were also stimulated by quinine to a great extent Using the nomenclature of Pfaffmann

(1941) the principal numbers of these fibres may be characterized as acid quinine fibres

Most of the non water fibres corresponded well to the fibres sensitive to salt described by Cohen *et al* (1955) and Sato (1963) These authors reported a relation between the sensitivity to salt and the absence of a response to water Pfaffmann s (1941) acid salt fibres seem to correspond well to the non water fibres predominantly sensitive to salt None of these authors reported a quinine and salt sensitivity in combination which is in agreement with this study Pfaffmann Cohen *et al* and to some extent Sato observed however that the salt sensitive fibres were stimulated by acids also In this respect their studies differ from the present one But both Pfaffmann and Cohen *et al* have stated a threshold value of pH 2 5 and lower for these fibres The acid used in this study had a pH of 3 1 which was higher than the mentioned threshold value This may explain the difference One fibre of the non water type was stimulated exclusively by quinine (Table I) and thus supports previous observations of the existence of specific ' quinine fibres (Cohen *et al* 1955)

The above observed relation in single fibres is indirectly supported by an observation in the glossopharyngeal nerve of the cat (Appelberg 1958) This author could not detect any response to water His recordings do however show that the response to 1 M NaCl was at least 3 times that of 0 2 M acetic acid This is striking as the results from the chorda tympani in this study revealed a stronger response to 0 05 M acetic acid than to 0 5 M NaCl Further there was always a response to water in the chorda tympani nerve These observations support indirectly the outlined relationship between a water sensitivity correlated to an acid sensitivity and a salt response related to the absence of a response to water

Some of the tested fibres both within the water and "non water groups responded to 0 5 M sucrose As shown earlier (Liljestrand and Zotterman 1954) sucrose solution and water can produce the same effect on water fibres Previous studies of the summated response to 0 5 M sucrose and water revealed a similar intense increase of the activity after alcohol (Hellekant 1965) A response to sucrose was however observed in some of the non water fibres This response can only be explained as a direct effect of sucrose (Fig 6) The fibre in this figure was stimulated to a lesser degree by 0 25 M sucrose (not included in the figure) The observation contrasts to a certain extent with those of Pfaffmann (1941) who reported a response to sugar only in one or two cases Pfaffmann did outline a tongue area possibly sensitive to sugar The existence of sucrose sensitive fibres in the cat was supported also by other observations Thus Liljestrand and Zotterman (1954) found a response to 0 6 M sucrose in Ringer s solution which eliminates a water effect since this is suppressed by the Ringer Thus direct stimulation by sucrose was indicated The statements of other authors (Beidler Fishman and Hardiman 1955) also indicate a taste response to sucrose in the cat though the response was weak

It is of course difficult to draw any conclusions on the gustatory effect of alcohol in man but perhaps a comparison could be ventured Renqvist (1920) noted the difficulty in defining the threshold of alcohol The slow increase of the recorded response observed in the whole chorda tympani both in man (Diamant *et al* 1963) and in cat dog and rat (Hellekant 1965) may explain Renqvist s observation Diamant *et al* (1963) reported that man does not possess fibres sensitive to water Their recording of the summated response in the chorda tympani in man did not show any initial decrease of activity to alcohol This supports the hypothesis that the initial decrease of activity to alcohol emanated from fibres sensitive to water The last mentioned authors also

compared alcohol of different concentration and its eventual taste similarity to the salt sweet sour and bitter qualities The subjects reports varied This is interesting as no correspondence was detected in this study between the sensitivity to any of the taste qualities and the sensitivity to alcohol in the taste units of the cat except that weak alcohol solutions increased the activity in the non water fibres more than in the water fibres

Diamant *et al* (1963) reported further that the eventual similarity to any of the conventional taste qualities ceased with increased concentration This may indicate a stimulation of all kinds of gustatory fibres and corresponds well with the observation obtained in the cat where all fibres mediating taste were stimulated at higher concentration

Since alcohol is very lipid and hydrosoluble a rapid penetration of the membranes by alcohol seems possible Renqvist (1920) considered that the alcohol penetrates the gustatory cells Other authors have expressed the same opinion from studies on other species (Dethier and Chadwick 1947) A penetration is also plausible in the cat The study on other receptors in the tongue may possibly contribute to a solution of the problem The possibility of a direct stimulation of the epithelial nerve fibres must also be taken into consideration According to previous authors (e g Adrian 1912) and my own observations alcohol applied directly to a nerve does not generate any action potentials but blocks the conduction Therefore the observed increase of the frequency must emanate from the receptor or its proximity

In an earlier study on the basis of observations from the whole chorda tympani it was suggested that the substantial response to water after alcohol emanated from fibres sensitive to water (Hellekant 1965) These fibres were also presumed to cause the observed initial decrease in the spontaneous activity when the tongue was exposed to alcohol The recorded response from separate fibres has proved this hypothesis to be true and the recordings indicate that the response to water after alcohol must be considered as a response to water

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From the Institute of Medical Physiology A University of Copenhagen Denmark

The Permeability of Brain Capillaries to Non-Electrolytes

By

CHRISTIAN CRONE

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Abstract

Crone C *The permeability of brain capillaries to non-electrolytes* Acta physiol scand 1965 64 407—417 — By means of the Indicator diffusion method blood brain barrier permeability characteristics were studied on dogs The permeability coefficient of fructose glycerol propylene glycol urea thiourea antipyrine ethanol propanol and butanol was calculated If these substances are ordered according to their rate of passage from blood into brain tissue a hierarchy is found which corresponds to that which is typical of the permeability of cell membranes to non-electrolytes. The experiments lend support to the view advanced by the late August Krogh *that the permeability characteristics of the blood brain barrier are analogous* to those of cell membranes in general This conclusion is discussed against the background of recent findings of morphologically-demonstrated tightness of cerebral capillaries which imply that material which diffuses from blood into brain tissue must pass the plasma membranes of the endothelial cells

The most common experimental procedure employed in investigations on the blood brain barrier has been to inject test substances intravascularly and then to cut out brain tissue slices at varying times after the injection and to determine the amount of material which has penetrated into the brain tissue Among the several shortcomings of the tissue analysis method an important one is that it permits very little detailed comparison of passage rates of substances which pass the blood brain barrier with any ease It is therefore not surprising to find the various test substances grouped only as having a *low* or a *high* ability to penetrate into brain tissue Obviously a method with a much higher time resolution is needed and the present article deals with attempts to apply the Indicator diffusion method (Crone 1963) to the transcapillary exchange processes in the brain This method permits an extremely high time resolution as the exchange processes in the capillaries are investigated within seconds after intraarterial injection of test substances

Krogh (1946) put forward the idea that the permeability characteristics of the blood brain barrier reflected those of cell membranes in general. One of the ways in which this statement could be tested was to clarify experimentally the importance of the polarity of non electrolytes for the rate of passage from blood into brain tissue. The purpose of the present study was to establish a hierarchy of a series of non-electrolytes according to their ability to pass from the blood into brain tissue and thus to define the physico-chemical factors which determine this ability. The following test substances were used: fructose, glycerol, propylene glycol, urea, thiourea, antipyrine, propanol, butanol, ethanol.

Theory

An account of how to convert a single injection method into a quantitative method for evaluation of transcapillary processes has been given previously (Crone 1963) and only a short description will be given here.

The method is based on the simultaneous injection of a non-diffusible reference substance (to indicate the dilution in the blood stream) and a test substance, the loss of which from the blood is to be determined. By comparing the concentrations of the two substances in the injected fluid with those found in individual blood samples collected every 2—3 sec from the superior sagittal sinus within 20—30 sec after the rapid intracarotid injection, the fractional loss of the test substance from the blood can be calculated. The advantage of the method is that the sampling takes place so soon after the injection that there can be no important build up of concentration outside the capillaries. And as the loss is determined during the early steeply rising part of the dilution curve it is presumed that the loss of test substance from the blood is proportional to the concentration in the capillaries. If this is true then it is possible to determine the relative speed with which a number of substances pass from blood into brain tissue. The fractional loss of a test substance is found as $(c_{ref} - c_{test})/c_{ref}$, where c_{ref} is the concentration of the non-diffusible reference substance and c_{test} is the concentration of the test substance. The concentrations are, of course, expressed relative to the concentrations of the substances in the injection solution. There is no direct proportionality between fractional loss (F) and the permeability coefficient (P), but a log relation exists

$$P = -\frac{\dot{Q}}{A} \ln(1 - F)$$

from which the permeability coefficient (cm×sec) can be calculated if the capillary surface area (A) and the rate of blood flow ($\dot{Q}$) is known. It is implicit in the method that the extraction is independent of the concentration of the test substance (which means that the loss of material during the passage through the capillaries is proportional to the intracapillary concentration). If the extraction is not independent of the concentration a mediated transfer mechanism is most probable.

Highly diffusible substances present a methodological problem, as the speed with which they pass into the brain is such as to permit equilibrium between the blood in the venous end of the capillary and the tissue supplied by that capillary. In such cases the Indicator diffusion method may underestimate the permeability of the rate limiting membrane and it must be admitted that the problem of how to differentiate between passage rates of highly diffusible substances is not solved by the Indicator diffusion method.

Methods

The experiments were performed on mongrel dogs anaesthetized with sodium barbitone (Mebumal) 25 mg/kg b w i v. Small dogs weighing about –12 kg were most suitable. The carotid artery on one side was cleaned and a small polythene catheter introduced into a side branch of the carotid artery, usually the superior thyroid artery. A Lucite adapter (Rodnight and Tresize 1957) was fitted into a hole drilled in the skull above the posterior part of the superior sagittal sinus near the torcula. Immediately before the injection of the test solution an incision was made in the sinus in order to collect blood leaving the brain without any

contamination by extracerebral blood The blood flowed freely and no suction was applied The free flo permitted the collection of 1/2—1 ml of blood every 2 sec The blood was collected in small test tubes mounted on a slowly moving kymograph An aliquot of the injection mixture was dissol ed in heparinized blood taken immed ately before the intracarot d injection In this way the composition of the injection mixture could be determ ned under circumstances similar to those which applied for the blood samples from the brain The determination of dye and of test substances was carried out on whole blood rather than on plasma in order to obviate corrections for loss of test substance into the erythrocytes

The volume of the fluid injected was 1—1 5 ml The injection lasted about 1—2 sec The tonicity of the injection mixture was always held very close to that of normal dog blood by addition of suitable amounts of NaCl

Evans Blue Dye was determined spectrophotometrically after dilution of the blood in saline

Fructose was determined by Bojesen s method (195?) Recoveries averaged 99 3 in experiments in wh ch fructose was added to whole blood

Glycer l p opylene glycol urea and thiourea were used as C-labelled compounds Glycerol and urea were delivered from The Radiochemical Centre Amersham England the other substances from New England Nuclear Corp Boston U S A

The radiactivity was determined on the supernatant after precip tation of blood with ZnSO NaOH by Somogyi s method. Radioactivity from C in urea and thiourea was d termined after wet oxidation according to van Slyke (1940) and van Slyke Plazin and Weisiger (1951) as modified by Sakami (1955) C-glycerol and C-propylene glycol underwent persulphate oxidation (Weinhouse 1949) The CO liberated was trapped in NaOH and plated as BaCO (Henriques *et l* 1946) Each plate was dried and the act v ty expressed as the activity per 16 mg BaCO f om an experimentally determined curve for self absorption of C in BaCO The samples were counted with equipment manufactured by Trace lab (TGC—14 Carbon Counter) Th background activity was 11 cpm The net activity in the majority of the samples was at least 10 times the background activity Every sample was plated in duplicate and the act vity expressed as the average of the activity of two plates 10 000 counts were registered The accuracy of the determination of radioactivity was tested several times by epeated analyses of samples from a large amount of supernatant The coeff cient of variation was found to be between 1 6 and 3 6 % The recovery of carbon atoms was determined by oxidation of each of the following substances urea (99 2) th ourea (97 8) glycerol and propylene glycol (98 3) — the figures in brackets showing the percentage recovery of ca bon

Ethanol propanol a d butanol were determined by means of the enzymatic method devised by Dotzauer *t al* (1957) The standard deviation as calculated from the difference between 20 dupl cate samples was for ethanol 0 75 mg for p opanol 0 69 mg and for butanol 0 98 mg

Antipyrine was determined by the method described by B od e *et al* (1949) as mod fied by Kraybill *et al* (1954) The recovery was 9? 3 on analyses on whol blood with a standard deviation of 0 50 mg (antipyrine added to a final concentration of 1? mg) The low recovery may be due to prote n bind ng of pa t of the antipyrine

The extract on ratio (E) of the test substances in the ind vidual samples was calculated as

$$E = 1 - (c_{es})_{rel} / (cT\text{—}18^{?}4)_{rel} \quad \text{where}$$

the suff x rel refers to the f ct that each concentrat on is exp essed elatively to the concentra t on of that particula substance in the inject on mixture

The ext act on r tios wh ch are cons dered n the next s ct on wer the calculated a erage of the extraction rat os as determined from the samples collected on the steeply ris ng part of the ind cator dilution urve (normally 2—4 samples were obtained from each experiment)

Results

1 *Fructose* It was found in an earl er investigation (Crone 1963) that the permeability of the brain capillaries to sucrose was zero or close to zero Fig 1 summarizes 13 expts in which the ability of fructose to pass into brain tissue was investigated The f gure shows the initial extraction of fructose in individual samples (zero time on the abscissae corresponds to the peak of the dilution curve — points before O time therefore lie on

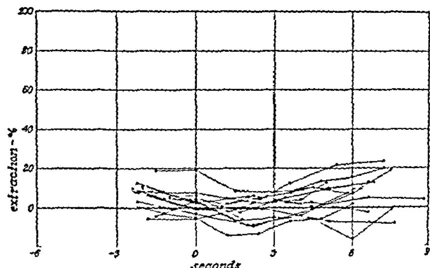


Fig 1 *Fructose experiments* The extraction (in per cent) of fructose during the first passage through the brain. Abscissae time of collection of blood from the superior sagittal sinus Zero-time corresponds to peak of dilution curve

the steeply rising part of the curve) During this interval the concentration of fructose outside the capillary is negligible compared with the internal concentration and the outward diffusion is assumed to be proportional to the mean intracapillary concentration The average extraction ratio calculated on samples collected before and including the peak sample was 0 041 (= 4 1 %)

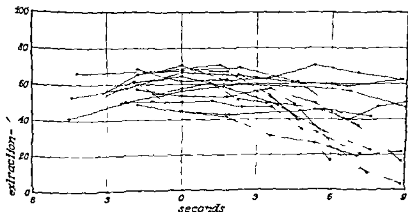
The occurrence of negative extraction ratios in the early period probably means that the extraction is so close to zero that analytical variations sometimes give negative values Negative extraction ratios after the peak may be due to net return of material to the blood

2 *Urea and thiourea* These two substances pass easily through most capillaries and distribute in the total water One would therefore expect a significant loss of these test substances from the blood during the passage through the brain On the other hand Collander and Barlund (1933) found a rather slow penetration into the interior of the Algae *Chara* and Overton (1902) had earlier found that the penetration into the interior of muscle cells took place only slowly According to Krogh's view one would therefore expect that the permeability of the brain capillaries to these substances was low

4 expts were performed using labelled compounds + inactive carrier) 2 with urea and 2 with thiourea The average extraction of urea was 0 11 (11 %) and of thiourea 0 07 (7 %) Fig 2 shows the results.

3 *Antipyrine* The molecular radius of this substance is 3 96 Å only a little less than the size of sucrose and a little more than that of urea The lipoid water partition coefficient is considerably higher than that of those substances and apparently this factor seems to be of importance as the average extraction of antipyrine in 15 expts was found to be 0 56 (56 %) Fig 3 summarizes the results from the individual experiments

Fig 2 *Lrea and th ourea experiments on brain* The upper part of the figure shows time-concentration curves for Evans Blue Dye and urea as obtained from samples collected from the superior sagittal sinus after injection into the common carotid artery Evans Blue Dye ○——○ Urea ●——● The lower part of the figure shows the extraction of urea (○——○ and ●——●) and of thiourea (×——× and +——+) in separate samples.



F g 3 *Initial extr ct on of antipyrine in the b a n* The extraction of ant pyrine n ind vidual samples collected from the superio sagittal s nus aft njection nto the common carotid artery Zero-t me correspond to the peak of th time-concentrat on curve.

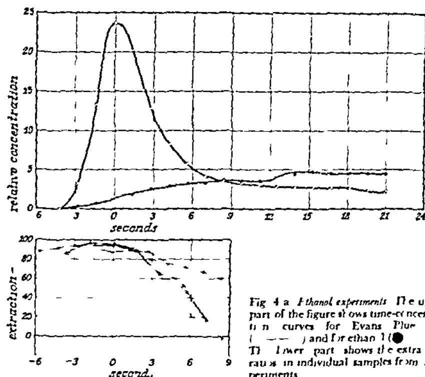


Fig. 4 a. *Ethanol experiments.* The upper part of the figure shows time-concentration curves for Evans Blue Dye (— — —) and for ethanol (● ●). The lower part shows the extraction ratios in individual samples from 3 experiments.

4. *Ethanol, propanol and butanol.* In order to examine *Krogh's* hypothesis further, the "lipoid-soluble" substances ethanol, propanol and butanol were studied.

According to *Collander's* investigations the increasing lipoid solubility of the molecules should dominate over the effect of increasing molecular size, so that one would expect a high extraction of all three substances in the brain. This was found in all experiments. The average extraction ratios were:

Ethanol 0.93 (93 %)
Propanol 0.90 (90 %)
Butanol 0.91 (91 %)

Fig. 4 a and 4 b show the results of individual experiments with ethanol and with butanol as test substance.

5. *Propanol, propylene glycol and glycerol.* These three substances represent another homologous series of non-electrolytes and they make it possible to study the effect of substituting H groups with OH-groups.

In order to get a clear picture of the effect of substitution, the substituted and the unsubstituted compounds were investigated in the same experiments, propanol being the representative of the unsubstituted molecule. Propylene glycol and glycerol were used as C-labelled compounds and were injected together with inactive carrier.

The experiments were perhaps the most spectacular of the whole series, because they showed the profound change of diffusibility through the brain capillaries caused by

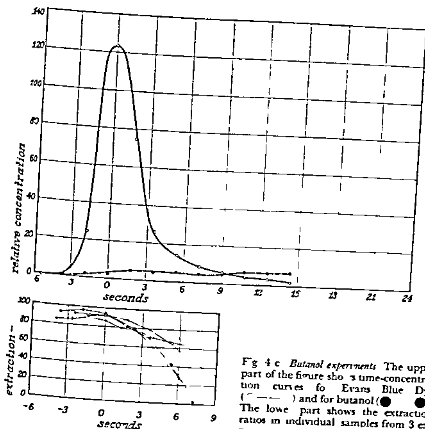


Fig 4 c *Butanol experiments* The upper part of the figure shows time-concentration curves for Evans Blue Dye (———) and for butanol (● ●) The lower part shows the extraction ratios in individual samples from 3 experiments

the introduction of one or two OH groups in the molecule The results are shown in Figs 5 a and 5 b where the curve for propanol should be compared with that of either propylene glycol or glycerol

The average extraction ratio of propylene glycol was 0 096 (9 6) and of glycerol 0 054 (5 4) It is worth noticing that a small molecule such as glycerol has a very poor ability to leave the blood during its passage through the brain and that this lack of diffusibility with great certainty can be ascribed to the presence of hydrogen bonding groups in the molecule

It is now possible on the basis of knowledge of the extraction ratios to make approximate calculations of the permeability coefficients of the brain capillaries to the various test substances employed The two unknowns in this calculation are the blood flow per unit of brain tissue and the average capillary area per unit of tissue Using the average figure for the cerebral blood flow 9 0 × 10 1 per sec per g tissue (Lassen 1959) and a capillary surface area calculated on the basis of counts of capillary densities in the brain 240 cm^2 per g of tissue (Crone 1963) and applying the formula given in the sect on "Theory" one obtains the permeability coefficients in Table I

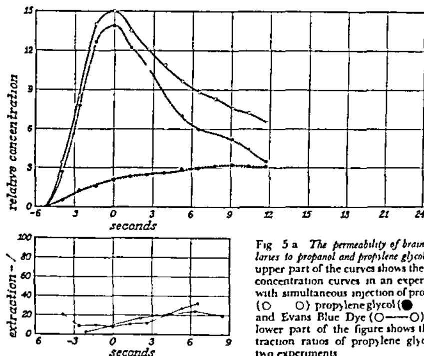


Fig 5 a *The permeability of brain capillaries to propanol and propylene glycol* The upper part of the curves shows the time concentration curves in an experiment with simultaneous injection of propanol (O O) propylene glycol (● ●) and Evans Blue Dye (O——O) The lower part of the figure shows the extraction ratios of propylene glycol in two experiments

TABLE I Permeability coefficients and initial extraction ratios for a series of non-electrolytes in brain capillaries

Substance	Initial extraction ratio	Permeability coefficient cm sec $^{-1}$
Inulin	0	0
Sucrose	0.004	0
Fructose	0.041	0.16 × 10
Glycerol	0.054	0.21 —
Thiourea	0.07	0.29 —
Propylene glycol	0.10	0.38 —
Urea	0.11	0.44 —
Antipyrine	0.56	3.3 —
Ethanol	0.93	10 —
Propanol	0.90	> 10 —
Butanol	0.91	10 —

[1] After Crone (1963)

[2] Probably underestimations because of methodological limitations (see text)

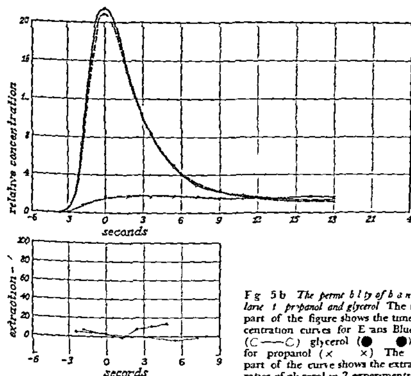


Fig 5b *The permeability of brain capillaries to propanol and glycerol* The upper part of the figure shows the time-concentration curves for Evans Blue Dye (C——C) glycerol (● ●) and for propanol (× ×) The lower part of the curve shows the extraction ratios of glycerol in 2 experiments

Discussion

The "Indicator diffusion" method permits a time resolution which makes it possible to distinguish between a number of substances with only small differences in rate of passage Thus it has been possible to draw a spectrum of non-electrolytes according to the transcapillary exchange rates The order of the substances in Table I corresponds to that for the same non-electrolytes found in the studies on permeability of cell membranes (Collander and Barlund 1933 Davson and Danielli 1952) There are small differences in the actual sequence of substances between different cell types but there is no doubt that the general pattern as known from studies on cell permeability also applies to the blood brain transfer The results of the present series of experiments thus support Krogh s general statement (1946) concerning exchange of material between blood and brain tissue see p 408

An interesting extension of the "cell membrane hypothesis" would be the demonstration of presence of mediated transport processes at this level The existence of mediated transport is just as typical of cellular exchange processes as the applicability of the Collander Barlund scheme Glucose was found to pass the blood brain barrier with a mechanism which is definitely different from that of the non-electrolytes mentioned in the present work and it was concluded that glucose transport into brain is an example of facilitated diffusion (or mediated carrier transport) Crone (1960 1961)

What is anatomical basis for the diffusion hindrance in the transfer of material between blood and brain tissue This question has been discussed ever since the slow exchange

was demonstrated and no definite answer can as yet be given There seem to be three relevant possibilities a) the capillary endothelial membrane may itself be of an unusually low permeability b) the glial foot processes outside the endothelial basal membrane might restrict diffusion and c) lack of interstitial fluid in the brain

The first viewpoint which has been supported over the years by Spatz (1931) Krogh (1946) Broman (1955) and Davson (1956) has gained considerable interest from recent electron microscopic investigations of the morphology of the endothelial cells lining the brain capillaries (Maynard Schultz and Pease 1957 Farquhar and Hartmann 1957) in which it was shown that the endothelial tube was completely continuous with no evidence of spaces between the individual endothelial cells and that overlapping of neighbouring cells was a typical feature Bennett Luft and Hampton (1959) place the cerebral capillaries in a category for themselves in a review dealing with known morphology of several different capillary areas in different organs

The possibility that the delayed exchange is due to non existence of an interstitial space in the brain has in recent years become a widely accepted explanation of the blood brain barrier phenomenon If the interstitial fluid is virtually non existent in the brain it is clear that exchange between blood and tissue is equal to exchange across cell membranes This could explain the common characteristics between exchanges in cells and exchange across the blood brain barrier It seems however to be forgotten that there is no unequivocal evidence for the postulated very small interstitial space On the contrary several investigators have found spaces of the same order of magnitude as that in muscle or even greater Rall Oppelt and Latlak (1962) found an inulin distribution space in the brain of 7—14 % when they perfused the subarachonoid space with inulin Davson and Spaziani (1959) found a sucrose space of about 16 % The theory of non-existence of an interstitial space in the brain rests essentially on electron microscopic studies of the brain but these morphological findings should not be accepted uncritically A serious objection is raised by van Harreveld and Schadé (1959) who claim that the cells in the central nervous system swell immediately after the circulation is arrested They find that the electrical impedance goes up signifying transfer of fluid from interstitial to intracellular phase whereby the interstitial volume goes down

As to the importance of the glial investments it might be relevant to point out that Lomerat in investigations on the cell dynamics in tissue cultures observed that glial foot processes were in continuous slow movement rather than in a fixed position (Lomerat 1952) If this movement also occurs *in situ* it means that the surface of the cerebral capillaries may be less completely covered than appears in histological sections The theory that the function of glial foot processes is to take up material from the blood and nourish the neurones by feeding these cells with the material thus absorbed (Dempsey 1958) has so far not received the necessary experimental support namely the demonstration that the main metabolic fuel glucose enters the glial cells before it enters the neurones

To conclude the present experimental results do not give any indication of to where the hindrance of diffusion between blood and brain tissue should be placed Whatever the localization may be it can be said that the relevant structure has many permeability characteristics in common with the plasma membranes in cells and it occurs to the author to be most reasonable to consider the possibility that blood brain barrier permeability reflects the permeability of the endothelial cells proper — in other words that the slow exchange of material in the brain is due to a very low permeability of the cerebral capillaries

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Acta physiol scand 1965 64 418—425

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Some Factors Regulating Normal Arylesterase Activity of Blood Plasma in Rats

By

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Abstract

Augustinsson K B and B Henricson *Some factors regulating normal arylesterase activity of blood plasma in rats* Acta physiol scand 1965 *64* 418—425 — A series of experiments were carried out in order to find the best conditions for further genetic studies on plasma arylesterase activity in rats Arylesterase was absent in the plasma at birth and adult activity levels were reached at the age of about 40 days Females had a higher activity than males The importance of technical details regarding narcosis blood sampling and handling of samples before esterase determination was investigated In rats starved for 42 hrs but otherwise untreated the plasma arylesterase activity increased about 40 per cent Refeeding of starved rats for 4 hrs before blood sampling decreased the activity The condition of momentary consumption of food greatly influenced the plasma arylesterase activity

The esterase pattern of rat plasma is highly complicated Three main types of esterases (aryl carboxyl and cholinesterases) are present (Augustinsson 1959) and in addition each type probably occurs in different forms with closely related substrate specificity or as isoenzymes (Augustinsson 1961) The arylesterase activity was found in at least two electrophoretically separable fractions and the total arylesterase activity measured with phenyl acetate differed greatly among individuals The original aim of the present investigation was to study the factors responsible for the variation between individuals particularly the genetic factors involved

The genetic control of plasma arylesterase activity in pigs has been demonstrated (Augustinsson and Olsson 1961) These animals contain in their plasma only one arylesterase the activity of which is fairly constant in adult animals when tested at various occasions Preliminary studies with rats however have shown that the arylesterase activity is considerably unstable which makes genetic studies difficult It was therefore necessary to study at first the factors responsible for this inconsistency in enzyme activity The purpose of this paper is to describe some of the experiments performed which determined the basis for an evaluation of the genetic control of arylesterase activity in rat plasma

TABLE I Arylesterase activity of blood plasma in a litter of rats Parents activity male 117 female 89 M (male) F (female)

Age in days	Rat number and arylesterase activity									
	3 F	5 F	6 M	7 F	8 F	9 M	10 M	11 F	13 M	15 M
53	117	147	118	127	114	96	96	123	79	130
96	195	162	107	165	164	134	120	148	140	108
128	175	179	137	160	102	120	126	209	154	138
137	207	223	130	170	149	165	13[illegible]	169	125	120
175			162	140	155			170	149	185
206			137	129	144			158	104	120
211			109	92	124			121	121	126
Mean value	172	178	129	140	136	129	119	157	125	132

Materials and methods

Animals All experiments were carried out on rats of a slightly inbred Wistar strain The weights of adult rats ranged from 175—250 g Except as otherwise stated the animals were allowed food and water *ad libitum* The following kinds of diets were used

Ordinary food A well balanced composition of laboratory food containing 80 of a mixture of cereals (mainly maize wheat and oats) 11 / fishmeal 6 % dried skim milk 1 fodder yeast 0 2 of a vitamin A and D mixture in granular form and 1 8 % salt mixture (McCollum)

Fatfree diet 68 5 % wheat starch 25 / dried skim milk 1 5 fodder yeast 2 A D mixture and 3 % salt mixture

In the diets with 3 or 10 / fat the corresponding amounts of starch in the fatfree diet were replaced by coco-nut fat

Blood plasma. Blood samples were collected with heparin in narcosis following intraperitoneal injection of a barbiturate (Mebumal) except as otherwise stated by puncture of the heart. In special cases blood samples were collected by puncture of the dorsal vein of the tail. The plasma was centrifuged free of all cells and used for esterase determination.

Esterase determination The esterase activity was measured by the Warburg technique at 25 C in a sodium bicarbonate CO_2 buffer of pH 7 4 containing 33 6 mM $NaHCO_3$ and 1 0 mM $CaCl_2$. Phenyl acetate in a final concentration of 10 mM as used as substrate Corrections were made for spontaneous hydrolysis of substrate and in certain cases with phenyl acetate as substrate for hydrolysis catalyzed by cholinesterase The determinations were then carried out in the presence of prostigmine bromid (final concentration 0 1 mM)

In determining the arylesterase activity of adult animals plasma was diluted 1:200 with the buffer solution and 0 4 ml of the diluted plasma was mixed with 1 6 ml of the substrate solution The esterase activity was expressed in μ moles of substrate hydrolyzed per min per ml plasma.

Results

Introductory experiments

In the preliminary mating experiments rats with different arylesterase (ArE) activity levels as judged by one determination were crossed The results of one of these crosses are illustrated in Table I In addition to a variation between individuals in the activity a considerable variation was also observed for each single individual from time to time

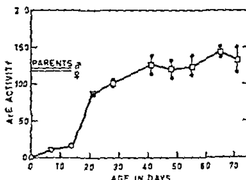


Fig 1 Arylesterase (ArE) activity of the plasmas of rats of the same litter at various ages Esterase activity (μmoles of substrate ml⁻¹ min⁻¹) measured with phenyl acetate Mean activity values of the parents are shown to the left. Mean activity values (□) and highest and lowest activity values (●) obtained on separate determinations with each sample Esterase activity at birth was measured with a pooled sample collected from the cord values at the age of 7 and 14 days refer to pooled samples (○) collected from two rats for each determination There was no significant sex difference in arylesterase activity of the three males and one female tested in this experiment.

In one individual (rat no 7) for example the ArE activity was 170 at 137 days of age and 92 in a sample taken at the age of 211 days Sex difference could also be observed in some cases females having higher activity than males Other experiments also demonstrated these variations within individuals in activity levels which make genetic studies almost impossible Variation between individuals in this activity is characteristic of several mammalian species However most species studied so far including humans show rather high stability regarding the ArE activity in adult individuals over a long period of time

Age variation in arylesterase activity

At birth the ArE activity of plasma was almost zero This was tested with a pooled sample collected from the umbilical cords The activity demonstrated with phenyl acetate as substrate was almost entirely due to the action of cholinesterase since in the plasma from cord blood the hydrolysis could be almost completely abolished by 0 1 mM prostigmine bromide After birth there was an increase of the ArE activity which reached adult activity levels at the age of about 40 days Fig 1 demonstrates the results of one of the experiments performed

Technical improvements in blood sampling and the role of narcosis

The importance of a number of technical details was studied regarding the blood sampling and further treatment of the samples before esterase determination After having altered some of these details final technique described above) it was found that technical errors could not be responsible for the unexpected inconsistency in esterase activity of the individual

Ether narcosis was used in the first experiments but was later replaced by mebumal narcosis The latter agent gave a more stable narcosis and blood sampling was made easier The degree of narcosis varied at different occasions for the same individual Table II shows that the ArE activity of the plasma was generally higher when the blood was taken from animals without narcosis but not always so Heart puncture gives rise to an appreciable trauma but results in obtaining a comparatively large volume of blood A comparison of this technique with that of puncturing a tail vein demonstrated (Table

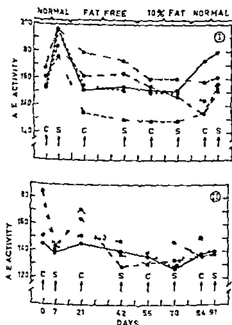


Fig 2

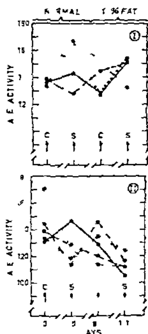


Fig 3

Fig 2 Effect of starvation and different diets on the plasma arylesterase (ArE) activity of female rats. Type of diet at top of diagram C Control values S in Fig 2I 42 hrs starvation before blood sampling S in Fig 2II 42 hrs starvation followed by food during 4 hrs before blood sampling

Fig 3 Effect of starvation and fat diet on the plasma arylesterase (ArE) activity of male rats. See Fig 2 for further explanations

TABLE II Effect of narcosis on the arylesterase activity of rat plasma

Blood samples taken	Arylesterase activity					
With narcosis	137	112	8[illegible]	146	89	111
Without narcosis	1[illegible]	131	13	197	1[illegible]9	13[illegible]

TABLE III Arylesterase activity of blood plasma obtained from five rats The samples were taken from the tail and the heart

Blood taken from the	Arylesterase activity				
Tail	1[illegible]8	114	144	146	110
Heart	133	114	109	1[illegible]	112

TABLE IV Arylesterase activity of blood plasma in rats after 6—8 hrs of starvation The activity

Age in days	F 134	F 135	F 136	M 137	M 138	F 139	M 140	M 141
	Rat number and arylesterase activity							
47	174	109	185	135	159	175	134	152
55	167	116	184	143	152	176	137	145
115	155	120	177	132	145	172	134	144
140	154	139	178	131	148	171	138	144
Mean value	163	121	181	137	151	174	136	147

Source of variation	D F	S S	M S	Ratio	P
Between sexes	1	6 364	6 364	5 06	<0 05
Within sexes	66	22 306			
Between rats	15	18 847	1 256 5	18 53	<0 001
Within rats	51	3 459	67 8		
Total	67	28 670			

III) no difference in the ArE activity between the 2 samples In these experiments the blood was first taken from the tail and then from the heart of the same animal The technical difficulties in getting enough blood (more than 1 ml) from the tail was the reason for using cardiac puncture throughout the main part of the investigation

After having revised various details in the procedure three litters were investigated from the age of 100 days The results obtained showed a less marked variation within individuals in ArE activity but this variation was still considerable and the main factor evidently remained unsolved

Role of feeding and starving

An indication of a factor related to the feeding of animals being of great significance for the ArE activity level in the plasma was revealed by the following observation On Sundays there was no one in charge of the animals which then had to use the food and water given them on Saturdays Part of the food had been refused and on Monday the animals consumed a large amount of fresh food When blood samples were collected on Mondays several animals were in a condition differing considerably from that of a normal feeding These facts seemed to be related to unexpected activity values obtained from samples taken on Mondays i e these values differed frequently from those taken on other days of the week

results from two litters have been pooled since the parents of both litters had medium esterase

F 145	F 146	F 147	M 148	F 149	F 150	F 151	F 152	F 153
153	148	140	106	121	154	158	183	154
139	149	136	109	118	147	150	183	154
149	157	170	105	148	157	160	189	156
144	158	167	113	153	163	164	156	160
146	153	153	108	135	155	158	185	156

In one experiment one group of animals was fed normal food *ad libitum* and another group with 60 % of calculated *ad libitum* consumption of normal food. For both these groups the variation in ArE activity was still significantly high and the amount of food consumed over a long time was therefore of minor importance.

The situation of momentary consumption of food in connection with blood sampling was found to be of the greatest importance as illustrated in Fig. 2. Ten female rats were starved for 42 hrs. Blood samples were then collected from half of the group (I in Fig. 2). The other 5 rats received food and after another 4 hrs blood samples were collected from them (II in Fig. 2). The results obtained are illustrated in the first part of the diagram in Fig. 2 ("Normal Diet"). The ArE activity increased markedly in the group of rats which were starved for 42 hrs and decreased in the rats which received food before blood sampling. In group II one of the rats died at the time of blood sampling.

Preliminary experiments revealed that the composition of diet also might play a significant role. The lipid component should be a factor of importance and a series of experiments were therefore performed feeding the animals with a diet without fat and diets containing 3 and 10 % fat respectively. A diet containing 10 % fat in most cases led to a marked decrease in ArE activity compared with diets without fat or with 3 % fat.

The effect of starving on the ArE activity was also studied when the animals were fed with different diets before the starvation. The results of these experiments are illustrated in Fig. 2 and 3. An increase in ArE activity as the result of starving was again obvious as was the effect of momentary consumption of food.

Experiments with stabilized arylesterase activity

After having realized the importance of the type and manner of feeding we let the animals starve 6—8 hrs before blood sampling. Table IV illustrates the results obtained with four activity determinations on samples taken from 17 rats of two litters out of parents all having medium ArE activity. Analysis of variance of the results demonstrated a high significance for differences between individuals in ArE activity. Sex difference

in this activity was also significant in spite of the relatively high variation between individuals. The basis for an evaluation of the genetic control of esterase activity levels now seems promising.

Discussion

In connection with the present investigation it is noticeable that starvation has been demonstrated to cause a change in plasma arylesterase activity. As the animals seem to react with varying activity values even in a normal feeding procedure, the best way of stabilizing the activity will be to introduce some hours of starvation.

Starvation generally causes a number of biochemical changes demonstrated with various animals and organs. This also concerns plasma enzyme activities. In rats, for instance, the following activities decreased: amylase (Soon Ju and Nasset 1959), alkaline phosphatase (Doose and Schmidt 1960), cholinesterase in adult female rats (Barrows and Chow 1958) and male rats (Soon Ju 1960), and clearing factor lipase of postheparin plasma (Monkhouse *et al.* 1961). An increase in enzyme activity as a response to starvation was found in rats for serum acid phosphatase (Laron and Kowadlo 1961) and for GOT and GPT in horses and cattle (Neuman and Maderová 1960). The meaning of these changes is not clear and in no cases they could be correlated with known biochemical or physiological reactions.

The biochemical background of the increase in arylesterase activity during the onset of starvation reported above is also obscure. Two recent observations may however be of some importance in relation to this change of the esterase activity level in blood plasma. First, it has been demonstrated by several authors (Goodman and Knobil 1959, Albrink and Neuwirth 1960, Corvilain *et al.* 1961) that both in humans and animals the concentration of plasma free fatty acids increased markedly as due to starvation. Mayes (1961) found a nearly threefold increase in this concentration following the transition of rats from the fed to the fasting condition. Secondly, it was demonstrated by Pilz (1964) that arylesterase of human serum in the presence of free fatty acids with a chain length greater than 6 C-atoms and aromatic acetates or propionates can trans-esterify to the esters of the long chain fatty acids (cf. Augustinsson 1964).

The physiological function of plasma arylesterases therefore may be their action in the transport of free fatty acids. The concentration in plasma is partly at least dependent on this esterase activity. We thus suggest a possible interrelationship between the increase in arylesterase activity observed in the present investigation and the previously demonstrated increase in free fatty acid concentration in rat plasma as due to starvation. Arylesterase may be a factor determining the transesterification between acetic acid and fatty acids of long carbon chains on the one hand and the free phenolic OH group of tyrosine. This amino acid is present in albumin to about 5 %, and albumin is known to bind fatty acids.

It might be possible to throw more light on these biochemical problems in connection with our genetic experiments. Animals with extreme activity levels, which can very likely be produced by selected matings, could possibly show some physiological response reflecting the biochemical action of plasma arylesterase.

This investigation was supported by grants from the Swedish Medical Research Council and the Swedish Natural Science Research Council. We are indebted to Mrs Inga Andersson, Mrs Ingrid Oppstedt and Mr Erik Johansson for skilled technical assistance.

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Acta physiol scand 1965 64 426—433

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Influence of the Thymus on Thyroxin-induced Changes in Blood Lymphocytes of Young Guinea-pigs

By

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Abstract

Ernstrom U and B Larsson *Influence of the thymus on thyroxin induced changes in blood lymphocytes of young guinea pigs* Acta physiol scand 1965 64 426—433 — Young male guinea pigs were thymectomized or sham-operated and treated with thyroxin or saline shortly after operation or one month after it The influence of thyroxin on the total number of blood lymphocytes and on blood lymphocytes with different mitochondrial content was compared in thymectomized and sham operated animals The average mitochondrial content of the blood lymphocytes was not influenced by thymectomy but was highly increased by thyroxin treatment in both sham-operated and thymectomized animals Thyroxin treatment decreased the number of lymphocytes/mm of blood in the thymectomized animals but not in the sham operated ones This reduction was due exclusively to a decreased number of lymphocytes with low mitochondrial content it was highly significant one month after thymectomy but not significant shortly after it Thyroxin treatment increased the absolute number of blood lymphocytes with a high mitochondrial content significantly in the sham-operated and almost significantly in the thymectomized animals It is concluded that an intact thymus is not necessary for a thyroxin induced increase in the number of blood lymphocytes with high mitochondrial content In addition the results suggest that thymectomy of young guinea pigs causes a failure of the thymo-lymphatic system to maintain an unchanged number of small lymphocytes/mm³ of blood during thyroxin treatment This failure is not manifested immediately after thymectomy but is significantly established one month after it

The lymphocytes in the blood of mammals are not a homogeneous population of cells Thus the existence of short lived and long lived lymphocytes (Ottesen 1954) and of lymphocytes with different sources and different destinations (Fichtelius 1953 1958) is reported Several morphological parameters such as cell size nuclear size basophilia of cytoplasm mitochondrial content and special inclusions have been used with the object of separating the blood lymphocytes into different classes with different origins or functions The number of mitochondria is probably a criterion of the metabolic activity of the individual lymphocytes Evidence also exists that the distribution of blood lymphocytes with respect to their mitochondrial content (MC) depends on functional

variation in the thymo-lymphatic system (refs see Ernstrom and Larsson 1961 and Discussion below) However the relation between the MC of the blood lymphocytes and the haemopoietic activity of the lymphatic organs is not precisely defined as is the correlation between the appearance of reticulocytes in the blood and erythropoiesis

Thyreogenous lymphatic hyperplasia in guinea pigs (Ernstrom and Gyllensten 1959) is accompanied by a great increase in the mean MC of the blood lymphocytes (Ernstrom and Larsson 1961) Determination of the mean MC of the lymphocytes of the thymus and the lymph nodes has not proved whether the lymphocytes rich in mitochondria are thymogenic or whether they derive from the lymphatic tissue (Ernstrom and Larsson 1963) This question is answered in the present investigation comprising a comparison between the effect of thyroxin on blood lymphocytes in thymectomized guinea pigs and in sham-operated controls In addition an account is given of an unexpected inverse effect of thyroxin on the number of blood lymphocytes in thymectomized and in sham operated animals

Material and methods

Young male guinea pigs weighing 180—250 g were divided into four groups

ST Sham-operated thyroxin treated
SC Sham-operated control treated
TT Thymectomized thyroxin treated
TC Thymectomized control treated

At 3—5 weeks of age the guinea pigs were thymectomized or sham-operated according to the technique used by Gyllensten (1953) Operation was performed under local anaesthesia (Xylocaine® Astra) Thyroxin (Thyroxine Nyegaard & Co A/S) was injected every 3rd day for 9 days (3 s c injections 50 μg/kg b w in the dorsum) The controls were given an equal volume of 0 9 % solution of NaCl The animals were killed by a blow on the neck on the 9th day after the start of thyroxin or control treatment

In one experiment (54 guinea pigs) thyroxin treatment was started 31 days after operation and continued to 40 days after it when the animals were killed

In a second experiment (68 guinea pigs) thyroxin treatment was started 3 days after operation and continued to 12 days after it when the animals were killed

Blood from the right ventricle was taken for supravital staining with neutral red and Janus green B according to the technique used in previous experiments (Ernstrom and Larsson 1961 1963) The slides with the blood lymphocytes stained supravitally were immediately examined in a light microscope (immersion objective 1 000 ×) and the mitochondrial content of 100 blood lymphocytes from each animal was determined In order to exclude subjective errors in the determinations the investigator did not know from which of the four experimental groups the samples were taken The lymphocytes were classified into 6 classes with respect to their MC i e lymphocytes containing 0—5 6—10 11—15 16—20 21—30 and > 30 mitochondria The average number of mitochondria/lymphocyte was calculated from the frequency of lymphocytes and the median of mitochondria in each class In the figures results and discussion only 3 categories of lymphocytes are compared lymphocytes with low (0—10 mitochondria/cell) medium (11—20) and high (> 20) MC

Differential counts of blood smears were made The total number of leucocytes/mm of blood was obtained by counting in a Burker chamber

The results were analyzed statistically by means of Student's *t* test

Results

Effect of thyroxin one month after thymectomy or sham operation

The relative distribution of blood lymphocytes as regards their *mitochondrial content* (MC) was almost identical in the sham-operated and thymectomized animals on the one hand and in the two groups of thyroxin treated animals on the other hand (Table I)

TABLE I Differential counts of blood lymphocytes in guinea pigs thyroxin treated or contro frequency of lymphocytes rich in mitochondria is demonstrated in the animals treate thymus

	Number of animals	Classification of lymphocyte phocytes in		
		0—5	6—10	11—1
Sham-operated thyroxin treated	13	5 0 ±0 4	31 6 ±1 2	36 4 ±1 [illegible]
Sham-operated control treated	13	19 3 ±1 5	44 1 ±1 2	23 4 ±1 5
Thymectomized thyroxin treated	14	7 6 ±0 6	34 9 ±2 0	34 0 ±1 6
Thymectomized control treated	14	19 7 ±1 6	44 1 ±1 7	23 4 ±1 5

Thus the average number of mitochondria/lymphocyte was not influenced by thymectomy but was highly significantly increased by thyroxin treatment in both sham operated and thymectomized animals

The *total number of lymphocytes mm³* blood was *decreased* in the thyroxin treated thymectomized animals when compared with thymectomized controls ($p < 0\ 01$) This decrease was confined to the lymphocytes with low MC ($p < 0\ 001$)

In the thyroxin treated sham-operated animals the total number of blood lymphocytes was *increased* when compared with sham-operated controls but *not significantly*. This increase was confined to the lymphocytes with medium and high MC ($p < 0\ 01$ and $p < 0\ 001$ respectively

The different results of thyroxin treatment on the number of blood lymphocytes in thymectomized animals and in animals with intact thymus is illustrated in Fig 1 and interpreted hypothetically in Table II In this table increased consumption of lymphocytes implies either increased lysis or an increased transformation to *other cell types* Increased production implies either an increased release of cells with or without increased mitotic activity in the lymphocytopoietic organs or an increased transformation from other cell types

Effect of thyroxin ... after thymectomy or sham operation

The relative distribution of blood lymphocytes as regards their MC in the four experimental groups was almost identical to that found one month after thymectomy or sham operation Table III

The total number of blood lymphocytes was not significantly changed in any experimental group The changes in the number of blood lymphocytes with low medium and high MC after thyroxin treatment were similar to those found one month after operation but showed less statistical significance Fig 2

treated one month after sham operation or thymectomy Mean ± standard error An increased with thyrox n compared to those not given thyroxin irrespective of the presence of an intact

according to their mitochondrial content Lym			Mean number of mitochondria/ lymphocyte	Difference
16—20	21—30	> 30		
15 3	9 1	2 6	13 4	3 5±0 3
±0 8	±0 6	±0 5	±0 2	p<0 001
7 9	3 8	0 5	9 8	
±0 7	±0 4	±0 2	±0 2	
14 1	8 0	1 4	12 5	2 7±0 4
±1 0	±0 5	±0 3	±0 2	p<0 001
8 1	4 1	0 4	9 8	
±0 8	±0 6	±0 2	±0 3	

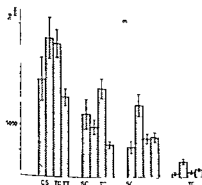


Fig 1

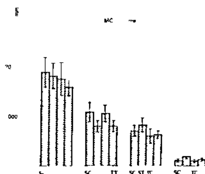


Fig 2

Fig 1 Total number of blood l mphocytes number with low (0—10 mitochondria cell) med um (11—20) and high (> 20) mitochondrial content in gu nea p gs thyroxin treated o control treated on month after ham operation o thymectomy A h ghly increased number of blood lymphocytes with high mitochondrial cont nt is demonstrated n the ham-operated an mals when treat d with thyroxin whereas a pronounced dec ease n th number of lymphocytes with low m tochondrial cont nt s demonstrated n the thymectomized thyroxin treated an mals. Mean ± standard error ST Sham-operated thyroxin treat d SC Sham-operated control treated TT Thymectomized thyroxin treated TC Thymectom zed control t eated

Fig 2 Total numbe of blood lymphocytes number with low med um and high m tochondrial content in guinea p gs thyrox n treated or control treated shortly after sham operation or thymectomy S milar changes ar demonstrated n sham-operated and thymectomized animals when treated w th thyrox n Mean ± tandard error Abbreviations as in Fig 1

TABLE II Summary of the thyroxin induced changes in the number of blood lymphocytes with low medium and high mitochondrial content in guinea pigs one month after sham operation or thymectomy ST Sham-operated thyroxin treated SC Sham-operated control treated TT Thymectomized thyroxin treated TC Thymectomized control treated

	Sham-operated animals		Thymectomized animals	
	Effect of thyroxin	Interpretation	Effect of thyroxin	Interpretation
Lymphocytes with low mitochondrial content	ST = SC	Increased consumption compensated by increased production	TT < TC $p < 0.001$	Increased consumption not compensated by increased production
Lymphocytes with medium mitochondrial content	ST > SC $p < 0.01$	Increased production	TT = TC	No increased production
Lymphocytes with high mitochondrial content	ST SC $p < 0.001$	Increased production	TT > TC p 0.05	Slightly increased production

TABLE III Differential counts of blood lymphocytes in guinea pigs thyroxin treated or con frequency of lymphocytes rich in mitochondria is demonstrated in the animals intact thymus

	Number of animals	Classification of lymphocytes in %		
		0–5	6–10	11–15
Sham-operated thyroxin treated	17	7 4 ±1 3	37 8 ±2 4	3[illegible] ±2 0
Sham-operated control treated	17	1[illegible] 1 ±1 5	42 9 ±1 7	25 9 ±1 3
Thymectomized thyroxin treated	1[illegible]	12 4 ±1 9	37 8 ±1 7	8.5 ±1 8
Thymectomized control treated	17	18 [illegible] ±1 2	44 1 ±1 2	21 1 ±1 3

The greatest difference between the experiments was seen in the effect of thyroxin treatment on the total number of blood lymphocytes in the thymectomized animals Thus thyroxin caused a decrease in the number of blood lymphocytes (— 11 % not significant) shortly after thymectomy and a greater decrease (— 38 % significant) one month after thymectomy The reduction in the number of blood lymphocytes was confined to the lymphocytes with low MC both shortly after thymectomy (— 24 % not significant) and one month after it (— 64 % highly significant)

Discussion

It might be argued that the thyroxin induced increase in the mean MC of the blood lymphocytes merely reflects the general change in metabolism of the hyperthyroid animals and not specific changes in the lymphocyte producing organs However a change in the MC of the blood lymphocytes is also seen when the activity of the lymphatic tissue is altered by other agents than by thyroxin injection *e g* by cortisone or antigens (for refs see Ernstrom and Larsson 1961) On the basis of such findings it has been suggested that the MC of the blood lymphocytes reflects the lymphocytopoietic activity of the lymphatic tissue in the body On the other hand Otani (1957) has suggested that the lymphocytes with high MC may derive from the thymus The present investigation has given some information relevant to this discussion

In guinea pigs treated with thyroxin shortly after thymectomy the increase in the number of lymphocytes with high MC was about the same as in sham-operated animals One month after thymectomy as well thyroxin treatment caused an almost significant increase in the number of lymphocytes with high MC, despite a decrease in the total number of blood lymphocytes These findings show that an increased number of blood lymphocytes rich in mitochondria appear in thyroxin treated animals even after thymectomy The MC of the individual lymphocyte is correlated to the size of the cell (Wiseman 1931 Fichtelius and Larsson 1961 Ernstrom and Larsson 1961)

trol treated shortly after sham operation or thymectomy Mean ± standard error An increased treated with thyroxin compared to those not given thyroxin irrespective of the presence of an

according to their mitochondrial content Lym			Mean number of mitochondria/ lymphocyte	Difference
16—20	21—30	> 30		
12 1	8 2	1 8	12 3	
±0 9	±0 7	±0 4	±0 4	1 7±0 5
10 6	4 5	1 0	10	p<0 01
±1 0	±0 7	±0 3	±0 4	
12 2	7 1	2 1	11 8	
±0 8	±0 7	±0 3	±0 4	1 6±0 5
8 0	4 4	1 2	10 2	p<0 01
±0 7	±0 5	±0 3	±0 3	

If the supposition that large lymphocytes (with high MC) differentiate to smaller lymphocytes (with lower MC) (Kindred 1955 Sainte Marie and Leblond 1958 Schooley *et al* 1959 Everett *et al* 1960 Yoffey *et al* 1961) is correct and applicable to the blood lymphocytes with different MC in the present investigation the thyroxin induced changes could reflect an increased production and/or release of large lymphocytes with high MC prior to an increased production of small lymphocytes with low MC. The latter cells did not in fact increase in number in the thyroxin treated guinea pigs — which might indicate a shortened life-span of the small blood lymphocytes in the hyperthyroid animals (increased lysis?)

On the other hand recent research seems to have proved that under certain experimental conditions small lymphocytes can increase in volume and become larger lymphocytes with a basophilic cytoplasm (Gowans *et al* 1960 Porter and Cooper 1962 a Rieke *et al* 1963) The thyroxin induced changes reported in this paper may therefore also reflect a decreased life span of small lymphocytes with low MC due to their transformation into larger lymphocytes with higher MC

Another alternative — even if more hypothetical than the previous ones — is that the large and small lymphocytes with high and low MC are not genetically related to each other but represent two different cell populations with different sources or functions In this case the increased number of blood lymphocytes with high MC and the reduced number of blood lymphocytes with low MC seen in the hyperthyroid animals are independant effects or effects related in other ways than by transformation of one type of cell to the other

In the present investigation an unexpected observation was that the total number of blood lymphocytes and of them only those with low MC was significantly reduced in the thyroxin treated thymectomized animals when compared to thymectomized controls Apparently the lymphatic organs of the thymectomized animals are incompetent to increase the production of lymphocytes with low MC (small lymphocytes) sufficiently to compensate for the consumption of such cells during thyroxin treatment (Table II This is compatible with the finding that thyroxin fails to induce lymphatic hyperplasia in thymectomized animals (Ernstrom 1965) Since the results are significant only when the guinea pigs are treated with thyroxin one month after thymectomy and not significant when treatment is given shortly after operation these effects of thymectomy seem to be time-dependent This failure of the lymphatic system of thymectomized guinea pigs to maintain the number of small lymphocytes/mm³ of blood unchanged during thyroxin treatment may be compared to the retarded regeneration of the lymphatic organs and the deficient recovery of the immunological reactivity of x irradiated or steroid treated animals observed after thymectomy even in adult age (for refs see Ernstrom and Gyllensten 1965) The results are in agreement with the hypothesis that the thymus is an important source of a cellular or humoral factor which seems to be of consequence for thyroxin stimulated lymphocytopoiesis

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On the Occurrence of Tyramine in the Rabbit Brain

By

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Abstract

Gunne L M and J Jonsson *On the occurrence of tyramine in the rabbit brain* Acta physiol scand 1965 *64* 434—438 — After chromatographic separation on a strong cation exchange resin no tyramine could be detected in an extract of 6 pooled rabbit brains This indicates that the whole brain content of tyramine must be below 10 ng/g wet tissue weight This observation does not support the concept that tyramine should be an important metabolic precursor of noradrenaline in the brain

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Tyramine (p-hydroxyphenylethylamine TA)[1] has been widely used as an experimental tool in order to induce a release of endogenously formed catecholamines (Burn 1960 Nasmyth 1960 A release is obtained at synaptic sites both in the peripheral adrenergic nerves and in the corresponding terminals within the brain Administration of TA thus activates adrenergic activity provided the catecholamine stores of the nerve terminals are not depleted of their catecholamine content

Recently Spector *et al* (1963) reported the presence of considerable amounts of TA in the mammalian brain This finding indicated that the decarboxylation of tyrosine might be a quantitatively important route in the biosynthesis of brain catecholamines Furthermore an important role of brain TA as an endogenous modulator of the catecholamine release seemed conceivable

According to the present investigation however the brain content of TA is low After chromatographic separation on a column of the strong cation exchange resin Amberlite CG 120 no TA could be detected with the sensitive fluorescence method of Oates (1961)

[1] Abbreviations TA = tyramine NA = noradrenaline NM = normetanephrine DA = dopamine MTA = 3-methoxytyramine 5HT = 5-hydroxytryptamine

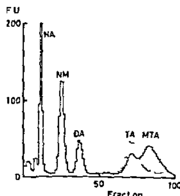


Fig 1 Separation on Amberlite CG 120 of a sample containing 10 µg each of noradrenaline (NA) normetanephrine (NM) dopamine (DA) tyramine (TA) and 3 methoxytyramine (MTA) Solid line direct fluorescence expressed as fluorescence units (F U) at 285/335 nm (act./fluor) wavelengths. Dotted line fluorescence at 470/570 nm after nitrosonaphtol condensation (Oates 1961)

Methods

12 freshly removed rabbit brains weighing together 110 g were pooled and homogenized in 600 ml of cold 0 4 N perchloric acid The homogenate was divided into 2 equal parts and to each part an addition of TA was made 1) The first part received 35 ng of tyramine C2—H (specific activity 140 000 counts/min) and 2) the second part was given an addition of 10 µg of TA — Each part of the pooled extract was centrifuged and filtered pH adjusted to 4 with 2N potassium hydroxide and after the potassium perchlorate had been spun down each extract was concentrated to dryness *in vacuo* The concentrated extract was dissolved in 10 ml of water and pH adjusted to 4 with 10 N sulphuric acid Each extract was then centrifuged in the cold and the supernatant was chromatographed on a column 100 × 4 mm of Amberlite CG 120 essentially according to Haggendal (1962) After passage of the extract the column was washed with 10 ml of water 10 ml of 1 N sodium acetate buffer pH 6 0 followed by 10 ml of water (the weak phosphate buffer of the original method did not remove some amphoteric compounds for instance tyrosine and 3 4-dihydroxyphenylalanine (dopa) while the strong buffer solution employed was effective in this respect) Elution was performed with 1 N hydrochloric acid and the eluate was collected in 1 3 ml fractions The fractions were analyzed for direct fluorescence at the wavelengths (act /fluor = 285/335 nm uncorrected instrumental values Aminco-Bowman spectrophotofluorometer) which were found to be optimal for authentic TA in hydrochloric acid

Pilot studies were undertaken to establish the relative R_f values of all amines expected to appear in the chromatogram Thus NA NM DA TA and MTA were added 10 µg of each to the same column Later the same experiment was repeated with the exception that only a small addition of TA was made in order to ascertain the minimal detectable amount of TA

For further identification besides the relative R_f values the peaks obtained by direct fluorescence were analyzed by specific fluorescence methods NA was identified by the trihydroxyindole (THI) method (*cf* Euler 1956 p 86) DA by the method of Carlsson and Waldeck (1958) and 5HT was recognized as a 5 hydroxyindolyl compound by its specific wavelength change in strong acid solution (*cf* Udenfriend 1962 p 170) The TA peak was identified by the 1 nitroso-2 naphtol condensation method of Oates (1961) and Spector *et al* (1963) using 0 5 ml of each fraction for the analysis The fluorescence was read at 470 570 nm

When TA H had been added to the sample the radioactivity of the fraction was estimated in a Packard Tri-Carb Liquid Scintillation Spectrometer 50 µl of each fraction were taken for analysis in a 7 3 toluene absolute ethanol solution containing 4 g of 2,5-diphenyloxazole and 100 mg of 1 4 bis 2(5 phenyloxazolyl) benzene per liter of toluene

Results

Fig 1 shows the direct fluorescence of a chromatogram of 10 µg of each of the following substances NA NM DA TA and MTA Although TA was not completely separated

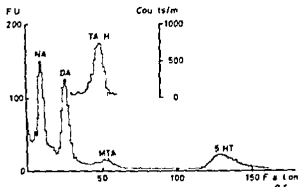


Fig. 2. Separation on Amberlite CG 120 of a pooled whole brain extract of rabbits (wet tissue weight 35 g). Direct fluorescence at 285/335 nm. The tyramine fractions were localized by addition of TA H³ 35 ng to the sample (upper curve, right scale).

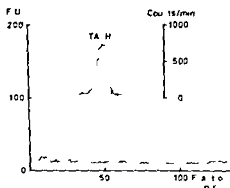


Fig. 3. Same fractions as Fig. 2 treated by the nitrosonaphtol condensation method and read at 470/570 nm.

from MTA the TA peak was readily identified by the nitrosonaphtol condensation method (dotted line in Fig. 1) which gives only a very low fluorescence with MTA. The recovery of TA was [illegible] per cent. It was found that the lowest amount of TA which could be detected by this method was 0.5 μg.

When brain extracts were chromatographed it was found that the high concentration of ions and the thorough washing procedure diminished the resolution capacity of the column to some extent. Fig. 2 and 3 present the data obtained from the pooled rabbit brain extract when 35 ng of TA H³ had been added. Fig. 2 gives the direct fluorescence where the NA, DA and 5HT peaks were later identified by special fluorescence methods. A small peak named MTA in Fig. 2 was obtained in the vicinity of the TA H³ scintillation peak (drawn above in Fig. 2 and 3). Fig. 3 shows the effect of treatment of the fractions by the nitrosonaphtol condensation method. It is obvious that the fractions containing the TA H³ activity do not correspond to any increase in fluorescence and thus that they do not contain fluorimetrically measurable amounts of TA.

When 10 μg of TA had been added to the pooled brain extract, however, fluorescence peaks appeared in the expected positions both by direct measurement (Fig. 4) and after treatment with nitrosonaphtol (Fig. 5). The fluorescence of the NA fractions determined by the THI method corresponded to approximately 11 μg in each half of the pooled extract giving a NA level [illegible] 0.2 μg/g wet tissue weight.

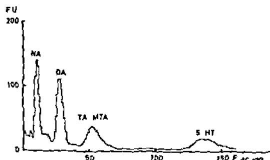


Fig 4

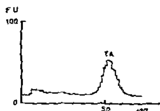


Fig 5

Fig 4 Separation on Amberlite CG 120 of a pooled whole brain extract of rabbits (wet tissue weight 55 g) Direct fluorescence at 285/335 nm 10 μg of tyramine added to the sample

Fig 5 Same fractions as Fig 4 treated by the nitrosonaphtol condensation method and read at 470/570 nm

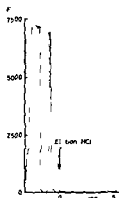


Fig 6 Fractions obtained prior to elution (at fraction O) from a pooled whole brain extract of rabbits (same sample as Figs 2 and 3 The fractions treated by the nitrosonaphtol condensation method and read at 470/570 nm.

Fig 6 shows the very strong fluorescence obtained in many fractions (nitrosonaphtol treated) prior to elution with hydrochloric acid (for details of the acid fractions see Fig 3) The fractions derived from the effluent of the brain extract had a fluorescence after nitrosonaphtol treatment around 7 000 fluorescence units while after 10 μg of TA the fluorescence of the eluate did not exceed 50 F U After the extract had passed the column there was a decrease in fluorescence but a second peak around 7 000 F U was obtained when the column was washed with buffer solution pH 6 0 Part of this second fluorescence peak may be accounted for by tyrosine which is known to be present in the brain in amounts of approximately 15 μg g (cf Udenfriend 1962 p 134) This amino acid has a fluorescence of approximately the same magnitude as TA after nitrosonaphtol treatment

Discussion

The present investigation originally aimed at a study of the possible role of endogenous brain TA as a mediator of adrenergic activity under physiological conditions and in

states of experimentally induced excitation where such activity is involved (*cf* Gunne 1963) The study of Spector *et al* (1963) indicated the presence of TA in the brains of various laboratory animals including rabbits as demonstrated by fluorimetric paper chromatographic gas chromatographic and enzymatic methods of identification Furthermore Creveling *et al* (1962) showed that administration of TA 2 C[14] gives rise to incorporation of C^{14} into urinary NA and NM indicating that TA *may* serve as a precursor of NA — In view of the data of the present investigation however the high tissue concentrations of TA obtained by Spector *et al* (1963) in various parts of the rabbit brain (cortex 1 3 cerebellum 1 8 midbrain 2 5 medulla 2 7 pons 1 1 caudate 1 0 μg/g other parts containing less) must be seriously questioned Although no figure was given for the whole brain TA concentration it is evident from the examples given that the whole brain level mu t be at least *l* μg/g The whole brain content of NA in rabbits averages 0 2—0 3 μg/g (Bertler and Rosengren 1959 own observations) Since the amount of NA in each pooled extract of the present study was 11 μg the expected TA content should be 33—55 μg

The absence of fluorimetrically measurable TA in the critical fractions indicates that the total amount of TA must have been lower than the minimal discernible 0 5 μg corresponding to 10 ng/g Thus the TA levels obtained by Spector *et al* (1963) were at least 100 times higher than those of the present study The reason for the high fluorescence obtained by these authors is obscure but it is evident from Fig 6 that there are highly fluorescent compounds within the brain which must not be allowed to interfere with the fluorimetric assay after nitrosonaphtol condensation

As a consequence of the above findings there remains some uncertainty regarding a hypothetical TA step in the biosynthesis of brain NA in the rabbit

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Effects of Ganglionic Blocking Drugs on Blood Glucose, Amino Acids, Free Fatty Acids and Catecholamines at Exercise in Man

By

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Abstract

Carlsten A J Haggendal B Hallgren R Jagenburg A Svanborg and L Werko *Effects of ganglionic blocking drugs on blood glucose amino acids free fatty acids and catecholamines at exercise in man* Acta physiol scand 1965 *64* 439—447 — The influence of exercise of submaximal load on the arterial concentration of catecholamines free fatty acids (FFA) and amino acids was studied in 6 young healthy men The noradrenaline (NA) level increased as a rule successively but the low adrenaline (A) level was unchanged After 14—18 min of work there was no change in the total FFA level but among the individual FFA the percentage of stearic and oleic acid decreased *whereas that of palmitic palmitoleic and linoleic acid increased The total amino nitrogen* level increased and among the individual amino acids there was a marked elevation in alanine When the exercise was performed during the infusion of ganglionic blocking drugs the increase in NA was abolished or less marked but the changes in the arterial concentrations of glucose lactic acid pyruvic acid amino acids and FFA were similar as without drugs These results indicate that the role of the sympathetic nervous system is of minor importance for the regulation of the free fatty acid level in serum during exercise

In exercise of submaximal strength and long duration the metabolism in the muscle is of aerobic type and the capacity of the oxygen transport and the oxygen extraction of the tissues are thus of prime importance The increased need of substrates during aerobic work can be covered by enhanced blood flow and increased uptake from the blood The uptake of substrates such as glucose lactic acid and free fatty acids (FFA) is at rest proportional to the concentrations in arterial blood (Goodale and Hackel 1953 Goodale Olson and Hackel 1959 Carlsten *et al* 1961 Scott Finkelstein and Spitzer 1962) at least as far as the myocardium concerns whereas amino acids are not or only to a limited extent oxidized by nonhepatic tissues (Elwyn *et al* 1956 Miller 1962)

TABLE I Noradrenaline in arterial plasma (μg/l)

	Without blockade			
	n	M	SD	M/SEM
At rest	6	0.135	0.075	4.35
2—4 min exercise	6	0.340	0.204	4.10
15—18 min exercise	6	0.497	0.348	3.50
24—26 min exercise	3	0.390	0.217	3.12
15 min after exercise	2	0.075	0.007	15.0

TABLE II Mean percentage composition of individual fatty acids within the FFA fraction and

		Without blockade			
		n	M	SD	M/SEM
$C_{16:0}$	I	6	23.45	1.938	
	II	6	1.53	1.211	3.10
$C_{16:1}$	I	6	4.12	0.668	
	II	6	0.917	1.096	2.05
$C_{18:0}$	I	6	12.8	2.275	
	II	6	−3.72	1.027	7.79
$C_{18:1}$	I	6	36.6	3.149	
	II	6	−1.70	2.033	2.05
$C_{18:2}$	I	6	8.33	1.457	
	II	6	1.52	1.801	2.06
Total FFA μM	I	6	628	200	
	II	6	−23.3	125	0.456

Abbreviations I = Values at rest II = The difference between values at exercise and at rest error of mean

The arterial levels of glucose and FFA are relatively unchanged during work (Carlsten *et al* 1962) except an initial fall in the FFA level in some individuals (Carlson and Pernow 1959 Friedberg *et al* 1960 Havel Naimark and Borchgrevink 1963) The myocardial uptake of FFA expressed in per cent of the arterial level seems to be the same at rest and during work (Carlsten *et al* 1961) As in the transport of oxygen the enhanced need of these substrates is thus covered by an increased blood flow per unit time

With arfonad				With vegolysen			
n	M	SD	M/SEM	n	M	SD	M/SEM
6	0.267	0.147	4.45	3	0.130	0.036	6.19
5	0.288	0.186	3.47	3	0.230	0.101	3.90
6	0.335	0.299	2.75	3	0.333	0.045	12.8
6	0.237	0.156	3.70	3	0.293	0.150	3.41
4	0.133	0.087	3.[illegible]5	3	0.187	0.042	7.79

mean total FFA level

With arfonad				With vegolysen			
n	M	SD	M/SEM	n	M	SD	M/SEM
6	22.4	1.85		3	21.8	2.15	
6	2.77	1.34	5.05	3	2.60	1.65	2.74
6	3.82	1.26		3	4.50	0.600	
6	1.13	0.543	5.11	3	1.10	1.311	1.45
6	14.18	2.98		3	10.8	2.07	
6	−3.13	1.38	5.55	3	−2.30	3.50	1.14
6	37.1	3.46		3	40.1	3.03	
6	−3.28	2.02	3.99	3	−1.83	2.08	1.52
6	8.88	0.873		3	10.70	1.59	
6	0.883	1.209	1.79	3	0.767	1.05	1.76
6	693	763		3	598	18.9	
6	−37.8	139	0.666	3	144	62.8	3.96

n = number of observations M = mean value SD = standard deviation SEM = standard

The consumption of glucose and FFA, the main sources of energy, is great in relation to the plasma pools of these substrates. An increased consumption in some tissues must rapidly be balanced by a release from others. The mechanism behind the regulation between input and output during exercise is not known.

One of the factors considered to be of importance in the regulation of the blood levels of several substrates is the symphathetic nervous system, the main transmitter substance of which is noradrenaline (NA). The sympathetic activity has been reported to be

TABLE III Amino acids in arterial plasma (mg/100 ml)

		Without blockade			
		n	M	SD	M/SEM
Threonine	I	3	1.70	0.186	
	II	3	−0.090	0.139	1.12
Proline	I	3	2.48	1.097	
	II	3	−0.130	0.452	0.50
Glycine	I	3	1.90	0.386	
	II	3	−0.060	0.416	0.25
Alanine	I	3	2.28	0.452	
	II	3	1.29	0.127	17.6
Valine	I	3	2.85	1.167	
	II	3	−0.133	0.516	0.45
Isoleucine	I	3	0.92	0.200	
	II	3	0.027	0.219	0.21
Leucine	I	3	1.56	0.437	
	II	3	0.120	0.331	0.63
Tyrosine	I	3	0.89	0.160	
	II	3	0.090	0.0954	1.63
Phenylalanine	I	3	0.88	0.311	
	II	3	0.027	0.239	0.19
Total NH N	I	3	2.94	0.210	
	II	3	0.223	0.0971	3.92

Abbreviation: See Table II.

involved in the mobilization of FFA from adipose tissues (Havel and Goldfien 1959, Bogdonoff, Weissler and Merrit 1960). Administration of NA as well as adrenaline (A) stimulates the releases of FFA from the adipose tissue (Dole 1956, Havel and Goldfien 1959, Bogdonoff, Weissler and Merrit 1960).

Presumably the effect of the sympathetic activity is of less importance in the regulation of the carbohydrate metabolism since injected NA in contrast to A has but slight influence upon the blood levels of glucose (Havel and Goldfien 1959). As judged from experiments in the rabbit NA has no effect upon the blood amino nitrogen level in contrast to A (Brunish and Luck 1952).

In the present study exercise of a constant submaximal load was performed by 6 young healthy men 22—28 years of age with and without administration of trimethaphan camphorsulfonate or hexamethonium bromide[1]. The arterial concentration of

[1] Trimethaphan camphorsulfonate (Arfonad, Roche) and Hexamethonium bromide (Vegolysen, May & Baker).

With arfonad				With vegolysen			
n	M	SD	M/SEM	n	M	SD	M/SEM
6	1.5?	0.259		3	1.57	0.238	
6	0.112	0.206	1.33	3	0.057	0.150	0.65
5	2.28	0.519		3	1.50	0.147	
5	0.132	0.40?	0.73	3	0.336	0.290	2.01
6	1.66	0.182		3	1.57	0.0473	
6	0.012	0.159	0.18	3	0.087	0.154	0.97
6	1.98	0.330		3	2.31	0.723	
6	1.553	0.460	8.27	3	1.630	0.512	5.50
6	2.88	0.575		3	2.57	0.440	
6	−0.045	0.432	0.26	3	0.237	0.320	1.28
6	0.87	0.067		3	0.95	0.0703	
6	−0.005	0.081	0.15	3	−0.077	0.0493	2.69
6	1.65	0.262		3	1.69	0.244	
6	0.050	0.127	0.97	3	−0.237	0.461	0.89
6	0.86	0.165		1	0.98		
6	−0.072	0.075	1.13	1	0.2?		
6	0.78	0.183		1	0.91		
6	0.160	0.222	1.77	1	0.05		
6	2.83	0.290		3	2.87	0.165	
6	0.215	0.216	2.44	3	0.290	0.0721	6.97

catecholamines glucose amino acids FFA lactic acid and pyruvic acids was determined The change in the symphathetic activity provoked was expected to elucidate some of the metabolic effects of the sympathetic nervous system

Methods and procedure

Chemical and physiological methods used have been previously described Carlsten *et al* 1962 Carlsten *et al* 1963 Hagendal 1963

Each individual was examined 2 or 3 times with intervals of 1—6 months under identical circumstances with except on for ganglionic blocking drug during the later examination

In a postabsorptive state the brachial artery was catheterized in the morning After a quarter of an hour s rest in the lying position a blood pressure and the oxygen consumption (Douglas bag) were measured followed by blood sampling from the arterial catheter With the subject still in the lying position the physical exercise was performed on a bicycle ergometer with a load of 600 kpm min (cases 1—5 or 800 kpm min (case 6 for 30 min and with frequent records of ECG and arterial pressure Oxygen consumption was determined after 12—17 min Blood for catecholamines was sampled ?—4 15 18 and 21— min after the start and 15

min after the end of the exercise whereas blood for the other analyses was collected between the 14th—18th min

Ganglionic blocking drugs were infused by vein The infusion rate was successively increased until a significant and constant effect on the blood pressure was achieved Some individuals were unable to fulfill the experiments because of muscle weakness headache and anxiety In these cases the experiments were later on repeated with a lower dose The mean dose of Arfonad was 0 8 mg/min and that of Vegolysen 1 6 mg/min

Results

Arterial oxygen saturation carbon dioxide tension and pH at rest and during exercise were not influenced by the ganglionic blocking drug The same fact applies to the oxygen concentration ventilation and respiratory quotient

The exercise load was submaximal In the studies without blockade the oxygen consumption was 5 3—7 2 times the rest value In all subjects the mean arterial blood pressure increased during exercise as an average with 16 mm Hg The increase in heart rate varied more between the subjects than the changes in oxygen consumption or blood pressure presumably due to differences in physical training The average heart rate at exercise was 127 beats/min the effect of exercise being an increase of 63 beats/min

When the exercise was performed during the infusion of ganglionic blocking drugs the increase in oxygen consumption was about the same as without drugs The increase in blood pressure was less marked than during work without blockers the mean arterial blood pressure rose as an average 6 mm Hg during Arfonad and 10 mm Hg during Vegolysen The increase in heart rate during exercise was somewhat greater with blockers than without The infusion of Arfonad induced an increase of the heart rate both at rest (20 beats/min $p < 0\ 02$) and at exercise (14 3 beats/min $p < 0\ 02$)

The respiratory quotient (RQ) increased during exercise from about 0 8 to the vicinity of 0 9 at both experimental conditions

The NA levels increased during exercise (Table I) Already after 2—4 min the arterial blood level was higher than at rest and increased as a rule successively during the exercise However there were great individual variations After exercise the NA levels decreased and had approached the basal levels within 15 min

During infusion of the ganglionic blocking drugs the NA levels at exercise in most cases showed only a slight increase or even decreased somewhat In 3 of the subjects there was a clear difference in the NA levels in relation to the administration of the drugs during exercise No apparent difference was found between the two ganglionic blocking drugs No measurable amounts of A were detectable in any of the experiments

After 14—18 min of work with or without administration of ganglionic blockers there was no significant change in the mean total FFA level (Table II) Among the individual FFA the percentage of stearic acid and oleic acid decreased and that of palmitic palmitoleic and linoleic acid increased at both experimental conditions Corresponding changes were observed in the absolute values except for palmitic acid which was unchanged

Physical work without and with ganglionic blockers provoked an elevation of the total plasma amino nitrogen and among the individual amino acids a marked elevation of the alanine level Table III

Ganglionic blockers did not influence the change in plasma concentration of glucose lactic acid or pyruvic acid provoked by the exercise

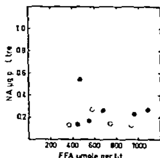


Fig 1 The plasma FFA level in correlation to the NA level at rest (○ ●) and during work (□ ■) without (○ □) and with (● ■) the infusion of arfonad

Discussion

Ganglionic blocking drugs interfere with the reflex adjustments of the circulation They block the vasoconstrictor pathways which control peripheral resistance and venous return Essentially they eliminate efferent nervous influences which keep blood pressure up especially when the subject is under such stresses as standing or exercise In the standing body position the hydrostatic redistribution of blood to the lower limbs is more exaggerated during ganglionic blockade inferring a disadvantageous reduction in the venous return to the heart with a decreased stroke volume as a result This hydrostatic effect is of course almost eliminated in the supine position Peripheral vasodilation and increased flow may occur *e g* in the limbs even in the absence of a rise in blood pressure (for example during exercise)

In the present investigation exercise was performed with the subjects supine in order to prevent unwanted blood pressure reduction when ganglionic blocking drugs were given Even with these precautions the dosage of drug had to be limited otherwise the subject had not been able to perform the amount of exercise prescribed That the level of infusion of drug was enough to cause marked ganglionic blocking effects was evidenced by several signs regarding the regulation of the circulation as well as from the central nervous system The individuals studied in the present investigation had higher heart rate both at rest and exercise less increase in blood pressure during exercise and cerebral symptoms when the drugs were given indicating a marked and as near complete ganglionic blocking effect as could possibly be achieved in this experimental setting Due to these effects of the ganglion blocking drugs the subjects complained of a feeling of muscular weakness some of them also of anxiety In a few experiments the subjects studied were not even able to continue the exercise for more than a few minutes because of these sensations In these subjects the experiment was repeated with a lower infusion rate on a later occasion

During work the increase in oxygen consumption and RQ was the same whether the subjects were given ganglionic blocking drugs or not Assuming that the combustion of amino acids is negligible this means that the drugs did not change the ratio oxidized carbohydrates to oxidized fatty acids Thus the blockers did not depress the combustion of fatty acids during work

Measurable amounts of A in plasma were not found in these experiments not even when the NA level increased considerably during exercise The increase of NA blood level at exerci is in agreement with the findings of Vendsalu (1960 who however

studied the effects of exercise of only short duration and with stepwise increase in load under which a successive increase of NA level was found With our procedure of a constant load but considerably longer duration the NA level increased progressively in 4 out of 6 subjects who all were in so-called circulatory steady state as judged from the constant heart rate and systemic arterial blood pressure There is evidence that not only the duration of exercise but also the degree of load has an influence on the NA level Further studies on this problem are in progress

The increase of the NA levels in blood during work was in some cases markedly reduced during the infusion of ganglionic blockers but in other cases there was no effect on the NA level The levels of NA thus varied considerably between the different subjects However the values of FFA amino acids and glucose lactic acid and pyruvic acid did not differ markedly whether the blockers had an effect on the NA levels or not No correlation was observed between the NA level and the FFA (Fig 1)

In an earlier report concerned with only few subjects it was found that the percentage composition of individual fatty acids changed during exercise the most obvious change was a decrease in the percentage of stearic acid (Carlsten *et al* 1962) This observation is confirmed by the present study The fall in the stearic acid level can depend on an increased inflow to the blood of fatty acids from the adipose tissue which is poor in stearic acid (Hirsch *et al* 1960) The reason to the less pronounced decrease in the level of oleic acid and increase in the levels of palmitoleic and linoleic acid can not be explained by any difference between the fatty acid composition in adipose tissue and in FFA These changes in the individual fatty acid levels during exercise were not influenced by the administration of the ganglionic blocking drugs

In the present investigation the ganglionic blocking drugs were given in the highest possible dosage with maintenance of muscular function and will to exercise This does not necessarily mean a complete blockade of all peripheral ganglia The sympathetic tone and release of NA may thus be normal or even above normal in certain localized areas *e g* fat tissue However it has never been definitely proved that the fat cells in the adipose tissues are innervated by adrenergic fibres With photo-fluorimetric technique such fibres have been found in the vessel walls and the majority of fat cells are apparently not in contact with the terminals containing NA (Wirsén 1964) The effect of increased sympathetic activity on FFA release might therefore depend on a diffusion of catechol amines from the vessel wall into plasma and into adipose tissue cells The present observation that infusion of ganglionic blockers which diminished the physiological increase in NA in the blood during exercise only to a very limited degree influenced upon the FFA level might indicate that the role of the sympathetic system in the regulation of the FFA level in plasma during exercise is of less importance compared to other FFA mobilizing mechanisms

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Acta physiol scand 1965 64 448—452

From the Aero-Medical Laboratory Institute of Physiology University of Lund Sweden

Determination of the Mixed Venous CO_2 Pressure with a Rebreathing Method

Correction for Volume Changes of the Lung Bag System

By

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Received 7 January 1965

Abstract

Lundin, G and D Thomson. *Determination of the mixed venous CO_2 pressure with a rebreathing method Correction for volume changes of the lung bag system* Acta physiol scand 1965 64 448—452 For determining the cardiac output with Fick s equation the mixed venous CO_2 content can be determined with a rebreathing technique During the rebreathing the nitrogen and CO_2 percentages in the bag were simultaneously registered by fast nitrogen and CO_2 meters This allowed the correction of the CO_2 values caused by volume changes of the lung rebreathing bag system due to the difference in O_2 uptake and CO_2 elimination When this correction is applied higher values for the cardiac output should be obtained

In a paper by Lundin and Thomson (1965) an analysis was made of the influence of rebreathing time on the determination of mixed venous blood CO_2 pressure when using a CO_2 rebreathing method for cardiac output measurement An almost significant difference of 0 6 mm CO_2 pressure was found between values obtained with the two rebreathing times used The values were higher with the longer time This difference was smaller than that reported by Hamilton (1962) who claimed it to be caused mainly by the CO_2 being dissolved in the fluids of the lung tissues When the CO_2 pressures of the mixed venous blood obtained by Lundin and Thomson were used to calculate the CO_2 content of this blood to be used in Fick s equation the values from the rebreathing time 13 sec gave about 5 per cent higher cardiac output than the values from the rebreathing time of 22 sec Hamilton found a 17 per cent higher cardiac output if the rebreathing time used to determine the mixed venous CO_2 was 16 instead of 24 sec Lundin and Thomson found it unlikely that the exponential course of CO_2 accumulation during rebreathing which was the basis for their graphical determination of the mixed venous CO_2 pressure was disturbed by the solubility of CO_2 in the lung tissues The difference however was large enough to be of importance and should if possible be corrected for In this paper we have therefore tried to analyze if such a difference could

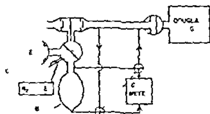


Fig. 1. Schematical drawing of the experimental arrangement.

be explained by volume changes caused by a gradually decreased CO_2 elimination from the blood and a constant oxygen uptake, a factor which should be more pronounced with a longer rebreathing time.

Method. Nine healthy subjects were used. On each subject 4 indoctrination experiments were done before the actual measurements started. In these experiments the CO was measured as described earlier by Jernérus, Lundin and Thomson (1963). The measurements this time also included simultaneous measurements of nitrogen and CO percentages of end tidal air and of the gas mixture in the rebreathing bag. The instruments for gas analysis were a rapid infrared CO meter (Godart Capnograph) and a fast response nitrogen meter (Lundin and Åkesson 1962). The sensitivity of the instruments allowed registration of changes in CO percentages of about 0.03 and 0.05 for the nitrogen. The CO meter which draws about 1.5 litres gas per minute was shunted to the breathing system. The nitrogen meter samples about 20 ml of gas per minute through the needle valve placed close to the mouth piece. This allowed the registration of the nitrogen percentages both in inspired and expired air. It was thus possible to control breath by breath the respiratory state of the subject. Fig. 1 is a schematical drawing of the experimental arrangements.

Experimental procedure

The seated subject, not necessarily being basal, was allowed to come into a steady state by resting about half an hour. After this time the subject was connected to the breathing system and started to breathe room air and the registration of the respiratory gases mentioned above started. *When the subject had come into a steady respiratory state with regard to the composition* of end tidal air the first rebreathing started from the rebreathing bag which was filled from the beginning with about 1 litre of gas mixture containing 2 CO and 35 O in nitrogen. The subject emptied the bag with each breath. After 11 breaths during which the CO and N in the bag had been recorded breath by breath, the subject was shifted back to breath room air. When a new steady respiratory state was obtained collection of expired air was started during which inspired and end tidal N and end tidal CO were continuously measured. After 6 min when this collection was finished the subject was again switched over to breathe from the rebreathing bag filled with the same gas mixture as before. The rebreathing rate this time was different from the first time. The rates used were 30 and 60 breaths per minute. The corresponding total rebreathing times for the eleven breaths registered were 22 and 13 sec. The breathing rate was governed by a metronome. The experiments started alternately with the slow or the faster breathing rate. On each subject 5 expts each with two rebreathing rates were performed.

Treatment of data

The percentage of CO *actually recorded with the CO meter are plotted in a linear coordinate* system with the first CO value on the abscisse against the second value on the ordinate. This makes the first point; the second point on the curve consist of values 2—3 and so on. Through all the points except the first two owing to incomplete mixing of lung bag air it is possible to draw a straight line and the intersection between this line and a line with the same distance from the ordinate and the abscisse should represent the point when the CO pressure in the alveolar air is the same as in the oxygenated mixed venous blood (Jernérus, Lundin and Thomson 1963).

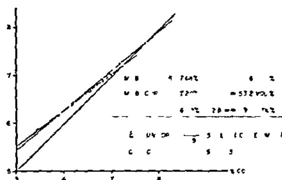


Fig 2 Graphical determination of the mixed venous CO_2 content with and without correction for volume changes Qc = Cardiac output/min

The nitrogen percentage in the rebreathing bag was used to calculate the relative volume change in the following way — After the first couple of breaths during which mixing between lung air and gas in the bag occurs there are 2 or 3 or more constant nitrogen percentages. We assumed that this means that no volume changes took place so far. The correction factors from the following volume decrease was calculated by dividing the constant nitrogen per cent just mentioned with the following nitrogen values. The CO_2 values were then multiplied with the corresponding correction factors and the corrected CO_2 values obtained were then plotted in the above mentioned coordinate system and a new line was drawn through the points. The intersection between this line and the equidistant line gave the corrected mixed venous CO_2. The arterial P_{CO_2} is assumed to be the same as the mean end tidal CO_2 values registered during the collecting period of 6 minutes and the eliminated CO_2 is determined from the expired air collected in the Douglas bag. For determination of the CO_2 content in arterial and mixed venous blood standard values (from Handbook of Respiration 1958) are used. No correction was made for the small amount of gas consumed by the nitrogen meter.

TABLE I

Frequency 30 breaths/min

	Uncorrected values			Corrected values		
	Mixed venous P_{CO_2}		Cardiac output	Mixed venous P_{CO_2}		Cardiac output
Subject	Mean	Range	Mean	Mean	Range	Mean
G L	51.2	49.[illegible]–53.3	2.6	48.2	47.2–49.9	3.1
D T	50.3	4[illegible].[illegible]–53.9	3.9	48.9	44.9–53.0	4.6
W P	51	50.2–54.3	2.8	49.9	48.1–51.4	3.1
B R	4[illegible]	4[illegible]–54.4	3.1	48.3	46.7–51.6	3.4
H H	49	44.6–52.3	[illegible].3	47.7	43.1–49.6	2.6
H W	45	43.0–46.1	2.9	44.1	42.3–45.4	3.0
D L	51	4[illegible].8–[illegible]	6.4	48.4	4[illegible].0–49.5	4.1
M L	5[illegible].9	[illegible]–54.3	3.5	51.2	49.[illegible]–52.5	4.1
M P	52.[illegible]	[illegible]0.5–55.6	4.4	50.0	49.2–51.0	5.[illegible]
Mean	50.3		3.[illegible]	48.4		3.7

Results

Fig 2 gives the values from a typical experiment Table I presents the values for mixed venous CO_2 pressure for the two breathing rates with both uncorrected and corrected values Before correction the mean mixed venous P_{CO} for the rebreathing rate 30/min was 50 3 mm and for the rate 50/min 48 7 mm or a difference of 1 6 mm Analysis of variance showed that this difference was highly significant The average cardiac output calculated using the above values were 3 2 l and 3 7 l respectively Thus a difference of 0 5 litre or a 13 % higher value with the rate 50/min When the P_{CO} values were corrected for the volume changes in lung bag system during rebreathing the P_{CO} for the rate 30/min decreased on an average 1 9 mm to 48 4 mm whereas for the rate 50/min the values decreased 0 3 mm also to 48 4 mm The cardiac output for the rate 50/min was unaffected by the volume correction The cardiac output for rate 30 became after correction 3 7 l or the same as the output determined from the rate 50/min both corrected and uncorrected

Discussion

By correcting the mixed venous P_{CO_2} values for volume changes of the lung bag system in the rebreathing method described we have found the same values with rebreathing rates of 30 and 50 breaths per minute The difference between the P_{CO_2} obtained with the two rebreathing rates was completely eliminated after the correction for volume changes which indicates that such changes explain the difference observed in the above earlier investigation by Lundin and Thomson (1964) and that solubility of CO_2 in lung tissue does not influence the mixed venous P_{CO} determined with this method

Frequency 50 breaths/min

Uncorrected values			Corrected values		
Mixed venous P_{CO_2}		Cardiac output	Mixed venous P_{CO_2}		Cardiac output
Mean	Range	Mean	Mean	Range	Mean
48 0	46 6—49 2	3 2	48 0	46 8—49 2	3 2
49 5	46 3—53 0	4 6	49 2	46 3—53 0	4 7
49 6	48 0—5? 1	3 2	49 4	48 0—51 0	3 2
48 4	45 7—52 2	3 3	48.	45 7—50 9	3 4
47 6	43 3—5? 3	? 6	47 3	43 3 50 9	2 6
44 8	41 4—47 3	3 0	44 6	41 4—47 3	3 0
49 2	48 5—50 6	3 9	47 8	46 9—48 5	4 3
51	49 2—53 1	4 0	51 4	49 ?—53 1	4 0
50 0	49 0—50 8	5	49 8	49 0—50 5	5 3
48 7		3 7	48.4		

The fact that the corrected mixed venous P_{CO} and cardiac output became the same with the two rebreathing rates also indicates that recirculation of CO_2 enriched blood in these resting experiments does not occur to a degree high enough to influence the P_{CO} obtained during the rebreathing time of 23 seconds used in the earlier publication by Jernérus *et al* (1963) It is however clear that values in earlier experiments include this volume error and that the resting cardiac output values therefore were somewhat too low

Our findings indicate that a rebreathing rate of 50 breaths per minute eliminates this volume error When the corrected values were compared with resting values for cardiac output determined with other methods we found that those obtained with the acetylene method came most close to our values whereas the values obtained with the direct Fick method were considerably higher and that also the dye dilution method gave higher values The cardiac index was 2 0 l for our subjects compared with 2 2 l for the acetylene method and 3 5 l for the direct Fick and Dye dilution methods (Handbook of Respiration 1959) It should however be pointed out that the values given above were obtained on reclining subjects whereas our measurements have been done on sitting subjects During work experiments the volume error should be more pronounced at least for higher working rates This error should however be counteracted by the fact that the end tidal P_{CO} during work is higher than the arterial P_{CO_2} (Asmussen and Nielsen 1956) When calculating the cardiac output we have assumed that on the healthy subjects used the end tidal P_{CO_2} equals the arterial P_{CO_2} The results from the above mentioned measurements by Jernérus *et al* (1963) using a rebreathing rate of 30 at rest and 50 during work showed a good agreement between cardiac output during work when compared with results obtained by other authors using both the acetylene and dye-dilution method (Asmussen and Nielsen 1953) This could mean that error in the determination of arterial and mixed venous P_{CO} during work in our earlier measurements were of the same magnitude Comparative simultaneous measurements using the dye-dilution and the described CO_2 method together with direct measurements of the arterial P_{CO} should however be made to further elucidate this question

The combination of a nitrogen and a CO_2 meter also made it possible to avoid the error of the CO_2 method due to a unsteady respiratory state The ease to apply these methods even an unlimited number of times should give it a considerable advantage over the direct Fick and Dye dilution methods

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Acta physiol scand 1965 64 453—462

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Vagal Relaxation of the Stomach

Experimental Re investigation of the Concept of the Transmission Mechanism

By

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Rece ved 9 January 1965

Abstract

Martinson J *Va al relax tion f the stoma h Exper mental re in estigat on of the concept of the transmission me hanism* Acta phys ol scand 1965 *64* 453—462 — The vagus nerves contain efferent fibres capable of relaxing the corpus and fundus of the stomach In experiments on cats this atropine resistant effect has been sho n to diff r in several respects fro n relaxation elicited by sympatheti stimulat on or infusion of catecholamines The vagal responses are more potent their latency is shorter and maximum response is attained at definitely lower frequencies The responses to sympathet c stimulation wears off much more rap dly after the end of stimulation than does vagally induced gastric relaxat on F nally the sympathetic or catecholami e gastric responses are more or less completely blocked by guanethidine or n tha l de which have hardly any effect on the vagal relaxation of the stomach Hexamethonium inhibits vagal relaxation and seems to be potentiated by atropine It s oncluded that the relaxation of the stomach on exc tat on of high threshold efferent vagal nerve fibres is me diated *via* preganglionic vagal f bres wh ch do not exert the r effect by any adren rgic mecha nism It is still not possible to say what peripheral mechanism is ctually respons ble for th s long lasting gastric elaxation

The time honoured observation that efferent vagus nerve stimulation is capable of inhibiting gastric motility (for references see McSwiney 1931) has recently been re investigated by Greeff Kasperat and Osswald (1962) Paton and Vane (1963) and by Martinson and Muren (1963) Working on isolated stomach preparations Greeff *et al* and Paton and Vane independently presented observations suggesting that such vagally induced inhibition is due to the presence of sympathetic nerve fibres in the vagus nerves This possibility was discussed in 1936 by Harrison and McSwiney who however at the same time expressed some doubt as to whether an adrenergic mecha nism ally was responsible for the vagal relaxation of the stomach for they had found the latency and type of the responses to differ in some respects from those induced by splanchnic nerve stimulation

Martinson and Muren (1963) showed that contraction of the stomach is induced by nerve fibres with a stimulation threshold differing clearly from that of the nerve fibres causing relaxation Jansson and Martinson (1965) who subjected the stomach to low continuous intraluminal pressure have shown that the relaxing vagal fibres possess powerful gastric volume augmenting properties suggesting that they are involved in the mechanism of receptive relaxation It has also been found (Martinson 1964) that the frequency response relationship of the relaxing effect of the vagal fibres is such that their action can hardly be ascribed to any overflow of *e g* an adrenergic vasomotor fibre transmitter, a mechanism which seems to be involved when *e g* a high frequency stimulation of adrenergic fibres is employed (*cf* Celander 1959 Kock 1959) Furthermore the latency of the gastric response to vagal stimulation is so short that it cannot be ascribed to any involvement of the adrenal medulla (Martinson and Muren 1963)

Preliminary experiments revealed that the gastric relaxation induced by vagal stimulation is not significantly affected by antiadrenergic agents such as reserpine guanethidine or α and β adrenergic blocking drugs This together with results obtained by Greeff *et al* and by Paton and Vane on isolated stomachs prompted the present study on the whole animal in which the effects of sympathetic and vagal stimulation and of infusion of catecholamines could be compared The results of these experiments were followed up by investigation of the effect of atropine and ganglionic blocking agents

Methods

Experiments were made on 36 cats weighing between 1 5 and 4 1 kg The animals were anaesthetized with chloralose 60—80 mg/kg b w administered i v after induction with ether After insertion of a tracheal cannula the cervical vagus nerves were dissected and divided The peripheral end of each nerve to be stimulated was placed in an annular bipolar silver electrode and connected to a square wave impulse generator (Grass Stimulator S4)

The abdominal cavity was opened by a midline incision In most experiments the intestines were removed this having been found to improve the reproducibility of the gastric responses A ligature was cautiously placed around the muscular coat of the abdominal part of the esophagus in such a way as to leave the vagal nerve trunks intact In a couple of cases the anterior vagal nerve trunk was dissected free and divided in order to permit direct stimulation at the subdiaphragmatic level

A polyvinyl wide bore catheter was introduced into the stomach cavity from the duodenal end and held in position by a ligature around the pylorus The catheter was connected to a volume reservoir filled with isotonic saline kept at 38 C This reservoir permitted continuous recording of gastric volume by a float recorder The height of the reservoir could be adjusted to keep the intragastric pressure constant at any desired level Only low intragastric pressures (2.5—4 cm of water) were applied A Perspex window was temporarily inserted in the abdominal wall during the course of some 10 experiments to permit visual inspection of the stomach (*cf* Jansson and Martinson 1965) Blood pressure was recorded with a mercury manometer connected to one femoral artery

The vagus nerves were stimulated in all experiments The stimuli used were such as to activate most of the nerve fibres causing relaxation of the stomach *i e* pulse duration was set at 2 msec and intensity at [illegible] volts (*cf* Martinson and Muren 1963) The impulse frequency was varied to obtain graded gastric responses

A geometric progression of impulse frequencies (1 2 4 8 and 16/sec) was used In many of the experiments the responses to only two of these frequencies (*e g* 1 and 4 imp/sec) were studied In most cases the responses to increasing stimulation frequencies were superimposed upon one another during the phase of recovery as shown in Fig 1 For further details see Results

After each stimulation series the intragastric pressure was set at zero and the stomach thereby allowing to empty But in those experiments where the rate of recovery was studied the level

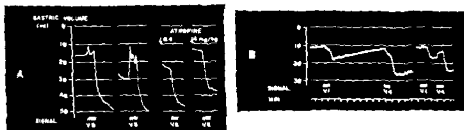


Fig 1 Gastric volume recordings from two experiments demonstrating general properties of the gastric relaxation responses to vagal stimulation (Markings at signal bars denote nerve [V = vagus] and pulse frequency [imp/sec]) Rhythmic alterations in volume are due to respiration

A Cat 2 6 kg Differences in immediate response during stimulation but relaxation to about same volume level at different initial volumes. After blocking the excitatory response to vagal stimulation with atropine the response develops rapidly and with short latency A high dose of atropine reduces response to some extent

B Cat 3 8 kg Recovery phase after vagal stimulation After stimulation with 4 imp/sec the intragastric volume reaches the same level either if it is started from basal resting tone or if it is superimposed on relaxation after 1 imp/sec

of the aforementioned reservoir was adjusted to keep the intragastric pressure constant Vagal stimulation was performed for exactly 1 minute at a time Both cervical vagi were stimulated simultaneously in most experiments

Sympathetic stimulation In 10 expts the sympathetic nerve supply to the stomach was prepared for stimulation The major splanchnic nerve on the left side was divided and its peripheral end was placed in a bipolar electrode similar to those used for the vagus nerves In 3 expts the nerves around the coeliac artery were prepared for stimulation The left adrenal gland was ligated in all these experiments and in 2 expts the right one also Stimulation was performed in essentially the same way as vagal stimulation As a rule a pulse duration of 5 msec was used at a voltage of 5 in order to activate all the efferent nerve fibres For comparison with vagal stimulation most stimulations were performed for 1 min

Infusion of drugs In 12 expts the splenic artery was dissected free near the hilus of the spleen after which a thin polyethene catheter was passed down to the coeliac artery Intra arterial infusions were given by means of a constant rate infusion pump Intravenous infusions were given in the same way *via* one of the femoral veins

Drugs Adrenaline (l base) noradrenaline (l base) and isopropylnoradrenalinesulphate were diluted in isotonic saline to a concentration of 10 μg/ml Other drugs used were Atropine sulphate (Merck) hexamethoniumchloride (Merck) guanethidine (Ismelin® Ciba) naphtyl isopropyl aminoethanolhydrochloride (nethalide Alderline® ICI) phenoxybenzamine (Dibenzyline® Smith Kline & French) Neostigmine methylsulphate (Neostigmin® Leo)

Results

Vagal stimulation The general appearance of the gastric response to vagal stimulation of the kind used in the present experiments is shown in Fig 1 All responses were obtained during a constant low intraluminal pressure The immediate response during vagal stimulation was a contraction when the initial volume was comparatively large i e when initial tone was low When tone was high (small initial volume) the volume response showed signs of rhythmic activity but nevertheless the stomach often relaxed during stimulation (see also Fig 2 and 3) Irrespective of the initial response however the stomach always relaxed after the end of stimulation and in most cases it attained several times its initial size Thus after relaxation was always a very long lasting re

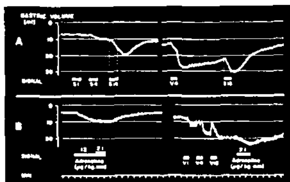


Fig 2 Gastric volume recordings demonstrating differences in responses to vagal and adrenergic stimuli

A Cat 3 8 kg Bilateral cervical vagal (V) and left major splanchnic (S) stimulation Note *slow initial phase and rapid regain* of volume after sympathetic stimulation as compared with response to vagal stimulation Note also dissociation of vagal and sympathetic recovery phases when proceeding simultaneously *B* Cat 2 8 kg Bilateral cervical vagal stimulation and infusion of adrenaline i a Note that even maximally obtainable adrenaline relaxation is small as compared with vagal relaxation but that adrenaline relaxation nevertheless can be added when stomach is almost maximally relaxed by vagal stimulation

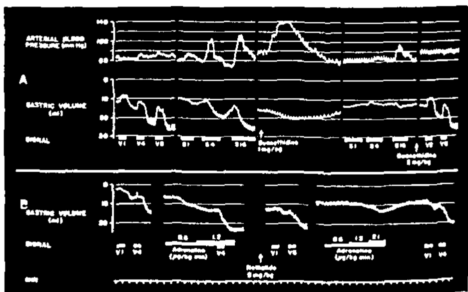


Fig 3 Effect of antiadrenergic drugs

A Cat 2 5 kg Gastric and blood pressure responses to sympathetic (left splanchnic) and vagal (intra abdominal ventral vagus) stimulation before and after guanethidine Note small blood pressure responses to this type of vagal stimulation

B Cat 2 9 kg Gastric response to adrenaline infused i a and vagal (bilateral cervical) stimulation before and after nethalide

axation which when maximal disappeared gradually within 15 to 20 min gastric volume decreasing at an average rate (n 11) of 0 8 ml/min (Fig 1 and 2) Not until the stomach had recovered its original size could relaxation of the same degree again be elicited This means that a constant initial volume was essential for the reproducibility of responses It was however possible to superimpose gradually increasin

TABLE I Gastric relaxation responses to sympathetic stimulation at frequencies 4 and 16 imp/sec and maximal responses to adrenaline compared with the vagally elicited relaxation at stimulation frequencies 1 and 4 imp/sec in the same animal

	Per cent of vagal response at frequency					
	1 imp/sec			4 imp/sec		
	%	SD	n	%	SD	n
Sympathetic 4/sec	91	85	5	35	23	5
Sympathetic 16/sec	190	114	5	79	25	6
Adrenaline i a 2—4 5 μg/kg min	130	49	6	57	20	6

stimulations upon each other as shown in Fig 1 The final gastric volume attained at the last stimulation of such a series was about the same as if this last stimulation had been performed alone from a resting initial volume The responses to the tested frequencies were very uniform with a mean of 77 % (Range 67—89) at 4 imp/sec and 94 % (Range 85—100) at 8 imp/sec of the response to 16 imp/sec found in a series of 10 stimulation series The relaxation was not in any respect dependent on the presence of a preceding contraction for when atropine had been given to block the excitatory nerve fibre responses relaxation started after a latency of 2—5 sec but otherwise showed the same characteristics (Fig 1)

Sympathetic stimulation and catecholamine infusion compared with vagal stimulation Sympathetic nerve stimulation caused essentially the same type and degree of relaxation of the stomach as did catecholamine infusion. The type and degree of the gastric relaxation produced by noradrenaline adrenaline and isopropylnoradrenaline were also largely the same On the other hand the relaxation induced by sympathetic stimulation and catecholamine infusion differed in many respects from that obtained by vagal stimulation The main differences are shown in Fig 2 and described below

1 Whereas the gastric relaxation response seen on vagal stimulation especially when atropine had been given (Fig 1 A) developed rapidly with a latency of only 2—5 sec the response to sympathetic stimulation developed slowly with a latency of 10—30 sec (Fig 2 A) Even when sympathetic stimulation was prolonged so as to allow the gastric volume to reach a stable level during stimulation (Fig 3 A) the responses to sympathetic stimulation were smaller than those to vagal stimulation Fig 3 A shows the by far most potent response to sympathetic stimulation in the present series In this very case the response to sympathetic stimulation 16 imp sec for 4 min amounted 90 % of the response to intraabdominal vagal stimulation with 16 imp/sec for 1 min The responses to catecholamines infused i a were still smaller than those to sympathetic stimulation when compared with the vagal responses Table I shows that the steady state relaxations induced by maximal or supramaximal doses of adrenaline were smaller than even the gastric response to vagal stimulation 4 imp sec

2 The vagal and sympathetic frequency response relationships were different This difference is demonstrated in Fig 4 Table I shows that vagal stimulation at 1 imp/sec was about equivalent to sympathetic stimulation with 4 imp sec and vagal 4 imp/sec equivalent to sympathetic 16 sec when stimulation time was the same (1 min)

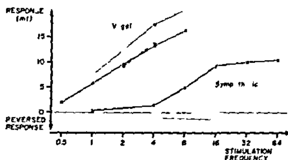


Fig 4 Cat 3 8 kg Diagramatic representation of gastric relaxation responses to vagal and sympathetic stimulation at different impulse frequencies in one experiment
Unbroken lines control
Broken l nes after guanethid ne 5 mg kg
Dotted lines after addition of nethalide 5 mg/kg

3 Vagally induced relaxation disappeared only slowly (Fig 1) while that produced by sympathetic stimulation or catecholamine infusion ceased much more rapidly (Fig 2) on the average (n = 11) 3 times as fast when calculated as volume units per unit time Furthermore (Fig 2) when a sympathetic stimulation or catecholamine infusion was applied during the recovery phase after vagal stimulation the rapid regain of volume after sympathetic stimulation proceeded as it seems dissociated from the slow recovery after vagal stimulation

4 It was often seen that although the stomach was seemingly maximally relaxed sympathetic stimulation or catecholamine infusion (Fig 2 B) could cause further relaxation Since the vagal relaxation was known to act mainly on the corpus and fundus (Jansson and Martinson 1965) it was assumed that the additional effect of catecholamine was due to an effect on the supposedly non relaxed pyloric antrum Visual inspection of the stomach through an abdominal window revealed that catecholamines and sympathetic stimulation not only relaxed the pyloric antrum but also substantially inhibited the peristaltic waves seen in the antrum during vagal stimulation When the stomach was relaxed as much as was possible by catecholamine infusion or sympathetic stimulation considerable further relaxation could be superimposed by vagal stimulation Sometimes this relaxation was of the same magnitude as the relaxation from normal basal level (Fig 3 B

5 The two types of responses differed markedly in sensitivity to antiadrenergic substances (see below

Effects of antiadrenergic drugs Guanethidine was used because of its ability to inhibit the release of the adrenergic transmitter at sympathetic nerve endings Phenoxybenzamine and nethalide were used to block α and β adrenergic receptors respectively The drugs themselves produced slight changes in the basal conditions of the smooth muscles of the stomach For instan e guanethidine produced a transient relaxation of the stomach within 15 30 min followed by contraction to its original or frequently to a smaller volume Fig 3 A This transient relaxation was in time correlated to the blood pressure rise produced by guanethidine Nethalide on the other hand produced much longer relaxations without any secondary tonicizing effect Nethalide sometimes relaxed the stomach t the same degree as t a infusion of catecholamines (which

TABLE II Effect of antiadrenergic drugs on gastric relaxation response to vagal and adrenergic stimuli. All values expressed as per cent of mean control response in one and the same animal. P values obtained by t test on difference between control response and response after administration of the drug

	Per cent of control after							
	Guanethidine 3 mg/kg				Nethalide 5 mg/kg			
		SD	n	P		SD	n	P
Vagal 1 imp/sec	86	38	9	0.30	72	12	4	< 0.02
Vagal 4 imp/sec	90	18	9	> 0.10	84	24	4	0.30
Sympathetic 4 imp/sec	0	0	4					
Sympathetic 16 imp/sec	7	6	5	< 0.001				
Adrenaline i.a. µg/kg min 0.5—1.5					13	19	5	< 0.001
Adrenaline i.a. µg/kg min 1.5—4.5					47	11	4	< 0.005

was the case in the experiment illustrated in Fig 3 B) Phenoxybenzamine which was used in 3 expts had only an insignificant effect on the basal tone of the stomach

The effects of guanethidine and nethalide on *vagally induced gastric relaxation* on the sympathetic inhibitory responses and on the inhibitory responses to adrenaline infusions are given in Fig 3 and 4 and in Table II Both guanethidine (average dose 4.5 mg/kg) and nethalide (5 mg/kg) markedly reduced the response to sympathetic stimulation and to catecholamine infusion respectively As shown in Fig 3 A a dose of 1 mg/kg of guanethidine was sufficient to abolish the gastric relaxation response to sympathetic stimulation although the vasoconstrictor response as seen in the blood pressure rise was not completely abolished In some experiments the gastric relaxation response to sympathetic stimulation was reversed to a slight contraction after adrenergic blockade (Fig 3 and 4) Table II and Fig 3 and 4 show that the vagal responses were on the average slightly reduced but not to a significant level by guanethidine (lowest dose 3 mg/kg) As regards the vagal responses after nethalide they were significantly weaker than in the controls at 1 imp/sec The total volume of the relaxed stomach (*i e basal volume + increase by vagal stimulation* was however increased by some 5—10 % after nethalide suggesting that the main explanation for the reduced responses after nethalide was the initially lowered tone When the intrinsic relaxing effect of nethalide was neutralized to some extent by the tonus increasing effect of guanethidine as it was in 2 expts one of which is represented in Fig 4 the responses to vagal stimulation were if anything increased as compared with control

Phenoxybenzamine was administered before nethalide in three of the experiments summarized in Table II (dose 0.5 0.8 and 1 mg/kg b w respectively) However as phenoxybenzamine affected neither the response to catecholamines (or to sympathetic stimulation) nor that to vagal stimulation the experiments were completed by administration of nethalide

Atropine and ganglion blockade The effect of atropine in different doses (0.1—10 mg/kg) was studied in 9 cats Moderate doses of atropine (0.1—0.5 mg/kg), which blocked

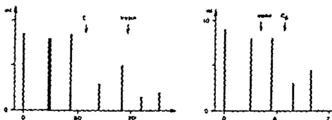


Fig 5 (Left) Cat 3 2 kg Sequence of gastric volume responses to vagal stimulation (4 imp/sec) before and after hexamethonium (C_6 — 5 mg/kg) and atropine (0 5 mg/kg) Note rapidly returning responses after C_6 and potentiating effect of atropine on block induced by C_6 (Right) Cat 2 7 kg Similar sequence of gastric volume responses to vagal stimulation (4 imp/sec) but with atropine (0 1 mg/kg) administered before C_6

the vagally induced excitatory responses were used in another 9 expts Atropine produced only small varying and insignificant changes in basal volume of the stomach

The gastric relaxation induced by vagal stimulation could be reduced by at most 50 % of control value by very large doses of atropine (1—10 mg/kg Fig 1) In 2 expts however a marked reduction was seen at the dose 0 1 mg/kg and in one of these the response to vagal stimulation was completely abolished by 0 35 mg/kg of atropine In these experiments the responses were regained with neostigmine (0 1—0 5 mg/kg)

The effect of hexamethonium (C_6) was studied in 11 expts In a dose of 5 mg/kg C_6 reduced the vagally induced gastric relaxation by some 50—80 % This blocking effect of C_6 was rather transient and the above mentioned reduction represents the mean values of the block within 15 min after injection of C_6 Fig 5 A gives volume responses to vagal stimulation with 4 imp/sec at different times in one experiment Atropine was added while the effect of C_6 was wearing off It can be seen that atropine potentiates the effect of C_6 A similar marked effect was noted in 5 out of 6 expts where C_6 (5 mg/kg) had been given before atropine (0 1—0 5 mg/kg) On the other hand it was not possible to show clearly any corresponding synergistic effect when atropine had been given before C_6 for the immediate inhibitory effect of C_6 on the gastric response was essentially the same whether atropine had been given previously or not Fig 5 B

Discussion

The present findings indicate that the vagally elicited relaxation of the corpus and fundus of the stomach is not mediated by any adrenergic mechanism This conclusion is based on the distinct differences found between the type of gastric response to vagal stimulation and to adrenergic stimuli and the sensitivity of the responses to antiadrenergic drugs

In agreement with the results of Brown McSwiney and Wadge (1930) the responses to splanchnic stimulation developed slowly after a relatively long latency disappeared comparatively rapidly and were prominent first at supraphysiologic stimulation frequencies As then an overflow of the adrenergic transmitter substance from e g the vasoconstrictor nerve fibre-endings is known to take place (Celander 1959 Kock 1959 and cf Brown 1960) these inhibitory responses may largely be due to such an unspecific mechanism

Vagal inhibitory fibres on the other hand seem to constitute a specific and powerful relaxing nerve fibre supply to the stomach It might be argued that these fibres can still be adrenergic the only difference between splanchnic and vagal adrenergic fibres being the type of effector they innervate This possibility however is not compatible with the gastric response to catecholamines as even large amounts can not mimic the vagal response characteristics in intensity or duration

Furthermore the failure of antiadrenergic drugs to affect the vagal responses while the sympathetic responses are greatly suppressed also strongly suggests that the vagal relaxation studied is not an adrenergic effect Recent studies by Abercrombie and Davies (1963) and by Black and Stephenson (1962) are referred to for further details concerning the general specificity and potency of the antiadrenergic drugs used

Greeff Kasperat and Osswald (1962) and Paton and Vane (1963) who used isolated stomach preparations showed that the relaxation of the stomach by vagal stimulation after previous administration of atropine was partly or completely inhibited by opilon and by brethylium (Greeff *et al*) or by TM 10 (used by Paton and Vane)

It is noteworthy however that in some of Paton and Vane s experiments periarterial sympathetic stimulation caused gastric relaxation which was completely inhibited by TM 10 while vagal and transmural stimulation sometimes caused responses which were only partially inhibited by the drug These latter observations seem to support the present findings but certain points possibly contributing to the discrepancies between the results of the present experiments and those of the authors referred to above might be mentioned. Greeff *et al* recorded only longitudinal movements of the stomach and the results might therefore not be strictly comparable with those obtained in the present investigation Paton and Vane recorded gastric volume at low intraluminal pressures a method principally similar to that used in present experiments and they also reported that vagal stimulation brought about gastric relaxation of the corpus and fundus and contraction of the antrum It therefore seems reasonable to conclude that the relaxing effect of vagal stimulation on the stomach in their experiments was the same as that noted in the present study It was noticed during the present experiments that rather extensive manipulation of the stomach which was sometimes necessary when preparing the sympathetic nerve supply or even seemingly less disturbing procedures as *e g* administration of artificial respiration were likely to considerably reduce the subsequent responses of the organ to vagal stimulation The responses to sympathetic stimulation under such circumstances seemed to be affected less It is therefore possible that the extensive preparation necessary for removing the stomach from an animal and placing it into an organ bath might have resulted in a modification of the experimental conditions Such a procedure will also considerably affect the normal nutritional supply of the smooth muscles

The most important difference however between the preparation used by the above mentioned authors *and that in the present experiments might be sought in the site of* nervous stimulation It is known that about 5 of all vagal fibres survive when the cervical vagus nerves are cut (Evans and Murray 1954) Many of these fibres are presumably adrenergic entering the vagus nerves within the thorax or below the diaphragm The systemic blood pressure rise often observed on intra abdominal vagal stimulation (Fig 3 A) suggests the precense of such fibres In the present experiments however with the stomach *in situ* the influence of these fibres on gastric volume must have been negligible as the vagally induced relaxation was not changed after guanethidine It can be assumed however that the preparations used by Greeff *et al* and by Paton and

Vane were in a condition as to favour the influence exerted by the vagal sympathetic fibres on account of the vagal relaxing fibre influence This assumption is in line with the above mentioned discrepant results on vagal and periarterial stimulation before and after TM 10 (Paton and Vane 1963 p 30 Fig 18)

Thus since observations made in the present experiments apparently rule out the possibility of an adrenergic transmission mechanism of vagal relaxation of the stomach another peripheral mechanism might be considered Atropine was examined for any blocking effect on the vagally induced relaxation in order to check whether the relaxation might not after all be brought about by some cholinergic mechanism The effect of hexamethonium suggested that the mechanism of vagally induced relaxation involves a ganglion transmission step The inconsistant effect of atropine alone in different animals together with the fact that atropine enhanced the blocking effect of hexamethonium suggests that atropine may here act on the ganglionic level rather than in the periphery

The peripheral mechanism actually responsible for the potent long lasting and fairly specific relaxation of the stomach muscles of the corpus and fundus is still obscure and requires further investigation If these fibres exert their action on the gastric smooth muscles by a local release of some specific transmitter agent it must be eliminated at a very low rate and it must be more potent than the catecholamines

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Acta physiol scand 1965 64 463—474

From the Department of Physiology University of Goteborg Sweden

Effects of Follicle Stimulating Hormone on Amino Acid Transport and Protein Biosynthesis in the Isolated Rat Ovary

By

KURT AHREN and LIDIA RUBINSTEIN[1]

Received 12 January 1965

Abstract

Ahren K. and L. Rubinstein *Effects of follicle stimulating hormone on amino acid transport and protein biosynthesis in the isolated rat ovary* Acta physiol scand 1965 64 463—474 — Whole ovaries from prepubertal rats were incubated in Krebs phosphate or bicarbonate buffer I v injection of follicle stimulating hormone (FSH) 4 hrs before removal of the ovaries stimulated the incorporation of glycine H into the ovarian protein and accelerated the intracellular accumulation of radioactivity from α aminoisobutyric acid C (AIB- C) glycine H and valine ^{14}C. Addition of FSH directly to the incubation medium did not have these effects The accumulation of amino acids was more rapid when the ovaries were incubated in bicarbonate buffer than when phosphate buffer was used Addition of glucose to the incubation medium did not influence the rate of accumulation of amino acids nor did it influence the effect of FSH Addition of puromycin to the medium inhibited and in higher concentration completely blocked the protein synthesis in the isolated ovaries FSH did however stimulate the accumulation of AIB- C glycine H and valine C even when the protein synthesis was completely blocked by puromycin It is therefore concluded that the effect of FSH on amino acid transport in the isolated rat ovary is not secondary to a stimulation of amino acid incorporation into protein and that this effect might represent a primary action of this gonadotrophin. Some evidence concerning the specificity of the observed effects of FSH is given

It is known in a general way that growth development and function of the ovary are controlled by gonadotrophic hormones from the anterior pituitary gland Present knowledge concerning the specific roles of the individual gonadotrophins in the different stages of ovarian development and function is however still very limited For the follicle stimulating hormone (FSH) one of the most prominent effects recognized with morphological methods is the stimulation by this hormone of mitotic proliferation of the granulosa cells and hence of follicular growth and maturation (e g Greep 1961)

[1] Fellow of the Consejo Nacional de Investigaciones (Argentina)

Vane were in a condition as to favour the influence exerted by the vagal sympathetic fibres on account of the vagal relaxing fibre influence. This assumption is in line with the above mentioned discrepant results on vagal and periarterial stimulation before and after I M 10 (Paton and Vane 1963 p 30 Fig 18).

Thus since observations made in the present experiments apparently rule out the possibility of an adrenergic transmission mechanism of vagal relaxation of the stomach another peripheral mechanism might be considered. Atropine was examined for any blocking effect on the vagally induced relaxation in order to check whether the relaxation might not after all be brought about by some cholinergic mechanism. The effect of hexamethonium suggested that the mechanism of vagally induced relaxation involves a ganglion transmission step. The inconsistant effect of atropine alone in different animals together with the fact that atropine enhanced the blocking effect of hexamethonium suggests that atropine may here act on the ganglionic level rather than in the periphery.

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ethylene gl col 6 naptalene 0 4 % PPO (2 5-diphenyl-oxazole) and 0 02 % POPOP (1 4 b s ?(4 methyl-5-phenyloxazolyl) benzene) To the aliquots of the media the same amount of TCA was added as that present in the aliquots of the tissue extracts Th d gree of quench ng was then found to be the same for the tissue extracts and their respecti e incubation media (in a first series of experiments this was tested by addition of known internal standards and recounting of the samples) and it was therefore not necessary to correct these counting data for quenching prior to the calculations of extracellular spaces and distribut on rat os (see belo)

For determining the radioactivity of the protein fractions (see below) the dried prote n was dissolved in 2 ml hydroxide of hyam ne (Packard Instrument Co) and 10 ml toluene containing 0 5 PPO and 0 0? % POPOP was added to each sample The samples were counted first without standard and then after addition of a known internal standard From the recovered disintegrat ons per minute the incorporated amount of radioactivity was calculated and exp essed as μmole of the valine C and glycin H respecti ely from wh ch it originated

M asurements of total and ext ac l lar tissue wa er

Total tissue water was determined by drying the ovaries to constant weight in an o en The val es are expressed as per cent of the wet tissu we ght (= ml water in 100 g tissue)

For determining the extracellular water the ovaries were incubated with 0 08 mM s icrose ^{14}C in the medium After the incubation the ovaries were homogenized in 1 ml 10 trichloroacet acid (TCA) and the percentage distribut on of ^{14}C in the o aries was determined by counting the radioactivity of appropriate aliquots of the prote n free TCA extract and of the media. The sucrose space of the ovaries as found to be the same after incubation times varying bet een 30 and 240 min indicating that the sucrose C equilibrated with the extracellular tissue flu d with n 30 min and that t d d not penetrate into the ntra ellular water

M ements of the intracellular ac umulation of AIB- C

The concentration of this model amino acid in the medium was 0 1 mM After the i icubation the ovaries were homogenized in 1 ml 10 % TCA and the radioact ity of the total tissu water was determined by counting aliquots of the protein free TCA extra ts The amount of AIB- C in the extracellular water was alculated ass ming that th concentration in the extracellular water was the same as in the incubation medium and this amount was subtracted from the total amount of AIB- C present in the tissue presumably gi ing a measure f the concentration of this amino acid in the intracellular compartment It has already been shown (Ahren and Kostyo 19 3) that this rad oactivity in the extract does represent AIB ince this am no acid is not metabolized to any extent by the rat ovary *in vitro* The uptake of AIB- C is expressed as the AIB- C distribution ratio (= cpm ml i tracellular water cpm ml ncubation medium) which ndicates the extent to wh ch this amino acid was concentrated by the cells

M su ements f the accumulation f gly ne H and valine C

F r both these amino acids the concentration in the medium was 0 1 mM After incubation, the ovaries were homogenized i 10 TCA and the ad oactu ty determ ned as described above for AIB- C. These amino acids can p obably be metabolized to som ext nt in the ovarian cells and t is ther fore likely that so ne of th radioacti ity in the tissue extracts d I not represent the original amino acids but arious metabolites f om them This has not been analyzed in the present experiments. It i ther for important to po nt out that th di tribu on ratios for these amino acids a e the ratios between the total ntrac llula adioacti ty and th rad activity in the incubation med um (= cpm ml int acellular wate cpm ml ncubat on medium)

M asurements f inc poration f glyci H and l ne C into pr tein

The method for determining the in orporation of these am no cids nt th o arian p ote n was v ry s m la to that described by Manchester and Y ung 1958 f measu ing ncorporat n f am no acids into the protein of th isolated rat diaphr gm After n ubati n the ovaries were homogen zed in 1 ml 10 TCA The precip tate was pun down and washed onc n 3 ml of 10 TCA The prec p tate was then heated f r 15 m n at 90 C in 3 ml f 10 TCA The insoluble ma erial was ext a ted with 3 0 ml f 0 4 normal NaOH and th inso l ble res d ie was r jected Th dissol ed protein was eprecip tated by the additi n of ml 10 TCA The precip tat was washed 3 t mes with ? ml d abs lute aceton an l once with th er and th n dryed n a acu um des cat The dryed prote n we gh ng 2—3 mg f r the poo led

6 ovaries was weighed on an analytical balance and the radioactivity of this fraction was determined as described above

The method for preparing the protein from the ovaries was tested in 7 expts (7 groups of 6 ovaries) by determining the nitrogen content (Kjeldahl's method) of the protein fractions. It was found that the per cent nitrogen in these dryed protein fractions varied only between 12.? and 1?.8

Measurements of concentration of AIB-¹⁴C in the follicular fluid

Attempts were first made to determine the concentration of AIB-¹⁴C in the follicular fluid of ovaries from prepubertal rats. These attempts were however not successful. Measurements of the concentration of AIB-¹⁴C in follicular fluid were therefore made in cystic rat ovaries produced by feeding the animals a diet containing 0.5 % thiouracil and injecting them with chorionic gonadotrophin (HCG) as described by Yatvin (1963). After injections of HCG (12 IU every second day) for 4 weeks these rats had ovaries with large follicular cysts. These ovaries were incubated for 2 hours in Krebs bicarbonate buffer containing 0.1 mM AIB-¹⁴C. The ovaries were then placed under a microscope and follicular fluid was collected in small glass capillaries which were weighed on an analytical balance before and after the puncture of the follicles. The filled capillary was crushed in a scintillation bottle containing 1 ml hydroxide of hyamine. The bottles were heated to 55 °C for 2—3 hours. 10 ml toluene containing PPO and POPOP was added as described above and the radioactivity was determined before and after addition of a known ¹⁴C-standard. The radioactivity of the incubation medium was also determined and the concentration of ¹⁴C in the follicular fluid was compared with that of the appropriate medium.

Results

1) Effect of FSH on accumulation of AIB-¹⁴C

Intravenous injection of FSH (500 μg/100 g b. w.) 4 hrs before removal of the ovaries increased significantly the *in vitro* accumulation of AIB-¹⁴C (Table 1). This was found when the ovaries were incubated in phosphate or bicarbonate buffer. The distribution ratios were however both for control and FSH-stimulated ovaries much higher when the ovaries were incubated in bicarbonate buffer (Table I). The effect of FSH was in addition more marked in the experiments with bicarbonate buffer. In all the following experiments which will be reported in this paper the bicarbonate buffer was used.

Addition of FSH *in vitro* in doses varying between 50 and 500 μg/ml medium did not influence the accumulation of AIB-¹⁴C.

6 expts were performed with the cystic ovaries from the thiouracil and HCG injected rats. The concentration of AIB-¹⁴C in the follicular fluid of these ovaries at the end of the incubation period varied between ?7 and 81 % of that found in the appropriate medium.

2) Effect of FSH on incorporation of glycine-³H and valine-¹⁴C

In vivo administration of FSH (500 μg/100 g b. w.) 4 hrs before removal of the ovaries stimulated significantly the *in vitro* incorporation of glycine-³H into the TCA precipitable protein of the ovaries (Table II). In the same type of experiments with valine-¹⁴C in the medium FSH had a slight but — with the number of experiments performed — not statistically significant effect (Table II).

3) Effect of FSH on accumulation of non TCA precipitable radioactivity from glycine-³H and valine-¹⁴C

It is seen from Table III that *in vivo* administration of FSH (500 μg/100 g b. w.) 4 hrs before removal of the ovaries significantly increased the *in vitro* accumulation of the

TABLE I Effect of i v injection of FSH on accumulation of AIB-¹ C b isolated rat ovaries incubated for 2 hrs in Krebs phosphate or bicarbonate buffer containing 5 5 mM glucose and 0 1 mM AIB C

Rats	Buffer	AIB ¹ C Distribution ratio		Significance of FSH effect
		Control	FSH	
SD	Phosphate	6 3±0 ᴗ4 (3)	8 5±0 ɔ? (3	p 0 0)
CR	Phosphate	5 8±0 3? (7	8 0±0 ɔ0 (7)	p 0 00ɔ
SD	B carbonate	14 1±1 29 (5)	¹1 0±0 /3 (5)	p 0 00)

¹ SD = Sprague Dawley strain CR = Charles River strain
Ratio of cpm/ml intracellular water cpm/ml medium
² Mean±S E Number of experiments in parenthesis
P values based on t test the procedure of paired data analysis ha been used
These data are from Ah en and Kostyo (196)

TABLE II Effect of i v inject on of FSH on incorporation of glycine H and val n C into protein of isolated rat ovaries incubated for 2 hrs in Krebs bicarbonate buffer containing 5 5 mM glucose

Amino acid in medium	Incorporation into protein (µmole g protein)		Significance of FSH effect
	Control	FSH	
0 1 mM glycine H	0 72±0 11 (9)	0 98±0 15 (9	p 0 0 ɔ
0 1 mM valine ¹ C	6 25±0 44 (6)	7 42±0 64 (6)	p 0 1

¹ Mean±S F Number of expe iments in parenthesi
P values based on t test the procedure of pa ed d ta nalysis has been used

non TCA precipitable radioact v ty both from glycine ³H and val ne ¹ C FSH did not show this effect when the for none was added *in vitro*

4) Effect of FSH on the a um ulati n of IIB ¹ C and glycine H in the absen e f glucose in the medium

In this series of experiments the ova ies were incubated with both AIB-¹ C 0 1 mM) and glycine ³H (0 1 mM in the medium The data in Table IV show that the effect of FSH on the *in vitr* accumulation of these two amino acids was the same whether glucose wa present n the mediu n or not

5) Influence of puromycin on pr te n ynthe is

Addition of puromy in to the in ubat on medium inhibited the incorporation of labelled amino acids into the protein of the isolated ovaries As shown in F 1 purom

TABLE III Effect of i.v. injection of FSH on accumulation of glycine-³H and valine-¹⁴C by isolated rat ovaries incubated for 2 hrs in Krebs bicarbonate buffer containing 5.5 mM glucose

Amino acid in medium	Distribution ratio[1]		Significance of FSH effect[3]
	Control[2]	FSH[2]	
0.1 mM glycine-³H	4.8±0.17 (9)	6.8±0.37 (9)	$p<0.001$
0.1 mM valine-¹⁴C	1.8±0.04 (6)	2.4±0.07 (6)	$p<0.001$

[1] Ratio of cpm/ml intracellular water : cpm/ml medium
[2] Mean ± S.E. Number of experiments in parenthesis
[3] P values based on t test, the procedure of paired data analysis has been used

TABLE IV Effect of i.v. injection of FSH on accumulation of AIB-¹⁴C and glycine-³H by isolated rat ovaries incubated for 2 hrs in presence or absence of 5.5 mM glucose in medium[1]

Ovaries	Number of experiments	Glucose in medium	Distribution ratio[2, 3]	
			AIB-¹⁴C	Glycine-³H
Control	5		12.5±0.48	4.3±0.21
FSH	[illegible]		17.5±0.57	5.9±0.49
Control	4		13.7±0.96	4.8±0.23
FSH	4	–	19.2±0.77	6.8±0.66

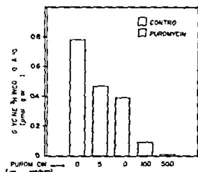
[1] Ovaries incubated in Krebs bicarbonate buffer containing 0.1 mM AIB-¹⁴C and 0.1 mM glycine-³H
[2] Ratio of cpm/ml intracellular water : cpm/ml medium
[3] Mean ± S.E. All the effects of FSH are statistically significant ($P<0.05$). P values based on t test, the procedure of paired data analysis has been used. Differences between corresponding values with and without glucose in medium are not statistically significant

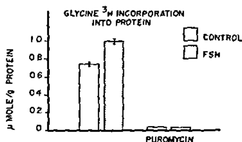
dihydrochloride in a concentration of 5 µg/ml medium significantly decreased the incorporation of glycine-³H. When puromycin was added in a concentration of 500 µg/ml medium, the incorporation was almost completely blocked (Fig. 1). It was therefore decided to use this last mentioned concentration of puromycin in order to analyse further whether FSH can also stimulate the transport of amino acids in the absence of an effect on the protein synthesis.

6) Effect of FSH on accumulation of amino acids in the absence of protein synthesis

Fig. 2 and Fig. 3 demonstrate that FSH (administered *in vivo* 4 hrs before removal of the ovaries) also stimulated the accumulation of AIB-¹⁴C, glycine-³H and valine-¹⁴C

Fig 1 Effects of various concentrations of puro mycin dihyd ochloride on the incorporation of glycine ³H nto the protein of isolated ovaries from prepubertal rats The ovaries were incubated for 2 hours in Krebs bicarbonate buffer containing 0 1 mM glycine H and 5 5 mM glucose





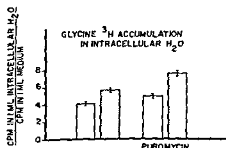
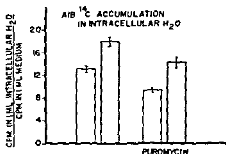


Fig 2 Influence of puromycin on the incorporation of glycine H into protein and o the accumulation of glycine- H and AIB- C by isolated ovaries from prepubertal rats The ovaries were incubated for 2 hours in Krebs bicarbonate buffer containing 0 1 mM AIB- C 0 1 mM glycine H and 5 5 mM glucose Puromycin d hydrochloride was added to the medium in a concentration of 500 μg/ml O ine FSH (1 mg ml in slightly alkaline 0 9 NaCl) was injected int a nously n a dose of 500 μg/100 g body weight 4 hours before removal of the ovaries Controls were injected with slightly alkaline 0 9 NaCl Standard error of the mean ndicated on the top of each column



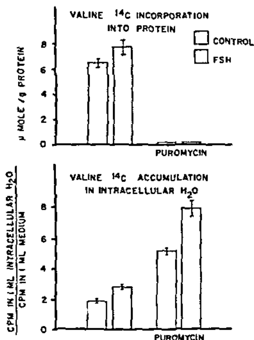


Fig. 3. Influence of puromycin on the incorporation into protein and accumulation in the intracellular water of valine ^{14}C by isolated ovaries from prepubertal rats. The ovaries were incubated for 2 hours in Krebs bicarbonate buffer containing 0.1 mM valine ^{14}C and 5.5 mM glucose. Puromycin dihydrochloride was added to the medium in a concentration of 500 µg/ml. Ovine FSH (1 mg/ml in slightly alkaline 0.9 % NaCl) was injected intravenously in a dose of 500 µg/100 g body weight 4 hours before removal of the ovaries. Controls were injected with slightly alkaline 0.9 % NaCl. Standard error of the mean indicated on the top of each column.

when the protein synthesis was blocked by puromycin and when therefore there was no effect of FSH on the protein synthesis. In the presence of puromycin the distribution ratios of glycine ^{3}H and valine ^{14}C were higher both in control ovaries and in ovaries from FSH injected rats than in the absence of this antibiotic (Fig. 2 and 3). The effect of FSH on the accumulation of these two utilizable amino acids was in addition more pronounced when puromycin was present in the medium.

The distribution ratio of AIB-^{14}C was lower when the ovaries were incubated with puromycin in the medium (Fig. 2). It can however be seen from Fig. 2 that the effect of FSH on the accumulation of this non utilizable amino acid was more pronounced when puromycin was present than when it was absent.

Specificity of the FSH-effect on the accumulation of AIB-^{14}C by the ovary

In one series of experiments intact rat diaphragms prepared according to Kipnis and Cori (1957) were incubated for 2 hrs in Krebs bicarbonate buffer containing 0.1 mM AIB-^{14}C and 5.5 mM glucose. There was no difference in distribution ratios of this non utilizable amino acid between diaphragms from control rats and diaphragms from rats injected with FSH 4 hrs before removal of the diaphragms.

In other series of experiments bovine growth hormone (STH) was injected intravenously in doses of 200–500 µg/100 g b. w. 4 hrs before removal of the ovaries. The ovaries were then incubated for 2 hrs in Krebs bicarbonate buffer containing 0.1 mM AIB-^{14}C and 5.5 mM glucose. No difference was seen in the AIB-^{14}C distribution ratios between ovaries from the STH injected rats and ovaries from simultaneously incubated ovaries from control rats.

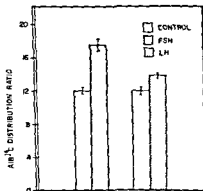


Fig 4 Comparison between the effects of FSH and LH on the accumulation of AIB-C by isolated ovaries from prepubertal rats The rats were injected with bovine FSH or ovine LH (500 µg/100 g body weight) in one intravenous dose 4 hours before removal of the ovaries The ovaries were incubated in Krebs bicarbonate buffer containing 0 1 mM AIB-C 0 1 mM glycine and 5 5 mM glucose Standard error of the mean is indicated on the top of each column P < 0 001 for the effect of FSH P < 0 05 for the effect of LH (P values based on t test the procedure of paired data analysis has been used)

Insulin was added to the incubation medium in doses varying between 0 001 and 0 1 IU/ml and the ovaries were incubated for 2 hrs in Krebs bicarbonate buffer containing 0 1 mM AIB-^{14}C and 5 5 mM glucose None of these doses of insulin however influenced the AIB-^{14}C distribution ratios of the ovaries

The effect of intravenous injection of LH was studied in 5 expts The hormone was given in a dose of 500 µg/100 g b w 4 hrs before removal of the ovaries The ovaries were incubated in Krebs bicarbonate buffer containing 0 1 mM AIB-^{14}C 0 1 mM glycine and 5 5 mM glucose and the AIB-^{14}C distribution ratio was determined after 2 hours of incubation As seen in Fig 4 the ovaries from the LH injected rats had slightly higher AIB ^{14}C distribution ratios than those of the controls This effect is statistically significant (0 05 > P > 0 02) This figure also shows however that the increase in AIB ^{14}C distribution ratio produced by LH was only about 30 % of that produced by the same dose of FSH under identical experimental conditions

Discussion

The pronounced ability of the isolated rat ovary to accumulate the non utilizable amino acid AIB was pointed out previously (Ahrén and Kostyo 1963) In that study the ovaries were incubated in Krebs phosphate buffer In the present study it was found that ovaries from prepubertal rats showed an even more pronounced accumulation of AIB ^{14}C when they were incubated in Krebs bicarbonate buffer It was also found that this concentrative uptake of AIB-^{14}C was not dependent upon the presence of glucose in the incubation medium and that the effect of FSH was also the same when the ovaries were incubated without glucose

In the experiments of Ahrén and Kostyo 1963 two possible sites of AIB-^{14}C accumulation in the ovary were discussed the intracellular compartment and the follicular fluid In the present study the concentration of AIB-^{14}C was determined in the follicular fluid from cystic ovaries After incubation of these ovaries the concentration of AIB-^{14}C in the follicular fluid was always found to be less than the concentration in the incubation medium These experiments with cystic ovaries of course can not prove that the same is true for the normal prepubertal ovaries which have very small follicles

but they do support the assumption that the pronounced accumulation of AIB-^{14}C in the prepubertal ovary is localized to the intracellular compartment and not to the follicular fluid

Some of the present results bear on the problem of specificity of the observed effects of FSH This hormone did not influence the AIB transport in the isolated rat diaphragm and insulin which stimulates strongly the AIB transport in the rat diaphragm (Kipnis and Noall 1958 Manchester and Young 1960) did not influence the AIB transport in the ovary Furthermore the hypophyseal growth hormone did not stimulate the AIB-transport in the ovary However injection of bovine LH produced a slight but significant stimulation of the AIB transport In the previous experiments of Ahrén and Kostyo (1963) injections of ovine or bovine LH had no significant effect on the ovarian AIB-transport It might be that a slight stimulation is more easily revealed when the ovaries are incubated in bicarbonate buffer as in the present study than when they are incubated in phosphate buffer as in the experiments of Ahrén and Kostyo

The slight effect of LH on the amino acid transport in the ovaries needs further experimental analysis before a final interpretation can be made In this analysis the following possibilities have to be kept in mind Firstly there might be a contamination of FSH in the LH preparation The LH preparation used is stated to have negligible FSH activity when tested with the augmentation test of Steelman and Pohley (Reichert 1962) but a small contamination of FSH can not yet be excluded Secondly the present experiments were performed on rats with intact pituitary glands It is therefore possible that the injection of LH produced a release of endogenous FSH and that this endogenously released FSH was the direct cause of the stimulation of the amino acid transport in the ovary Thirdly it is possible that LH enhanced the accumulation of amino acids in ovarian cells other than those stimulated by FSH Finally there might be only quantitative differences between the effects of FSH and LH and thus a real overlap in actions of these two gonadotrophins

The fact that FSH accelerated the rate of incorporation of glycine-^{3}H into ovarian protein shows that one of the very early effects of FSH is to accelerate the protein biosynthesis in at least some of the ovarian cells

In all the experiments with valine-^{14}C slightly higher incorporation into protein occurred in ovaries from FSH treated rats than in ovaries from control rats This effect was with the limited number of experiments performed not statistical significant This result however does not exclude the possibility that an effect of FSH can be demonstrated under slightly modified experimental conditions It might be that the concentration of valine-^{14}C 0.1 mM in the incubation medium was too high to allow a more marked effect of FSH It is known from other systems *e g* from experiments with Ehrlich mouse ascites tumor cells Riggs and Walker 1963) that the rate of incorporation of a certain amino into protein of isolated cells is dependent upon the concentration of this amino acid in the incubation medium and that the incorporation is saturated when the level of the added amino acid becomes great enough This point of saturation is also known to be different for each amino acid in addition different types of cells vary considerably in their ability to incorporate a given amino acid

In the experiments with glycine-^{3}H and valine-^{14}C the accumulation of non TCA precipitable radioactivity in the ovaries at the end of the incubation period was clearly increased by the FSH treatment This result illustrates that FSH can stimulate the uptake not only of the non utilizable amino acid AIB but also of normal utilizable amino acids

One important question in the interpretation of these results is whether the effect of FSH on amino acid transport in the rat ovary is a primary direct one or the increased uptake of amino acids is secondarily induced by the effect of FSH on stimulating the rate of intracellular protein synthesis Puromycin which is known to be a specific inhibitor of protein biosynthesis at the ribosomal level (Yarmolinsky and de la Haba 1959 Rabinovitz and Fisher 1962) was used in an attempt to analyse this question This antibiotic added to the medium inhibited and when used in higher concentration completely blocked the protein synthesis in the ovarian cell It was found that FSH stimulated the accumulation of AIB-^{14}C and the accumulation of TCA soluble radioactivity from glycine ^{3}H and valine ^{14}C even when the protein synthesis was completely blocked This result demonstrates clearly that the effect of FSH on the amino acid transport in the rat ovary is not secondary to the stimulation of amino acid incorporation into protein This result cannot of course be taken as evidence that FSH increases protein synthesis in the ovarian cells solely by this influence on amino acid accumulation

In this connection a comparison with observations on the isolated rat diaphragm is of interest In that tissue it has been found that insulin and under certain conditions growth hormone enhances the cellular accumulation of amino acids this effect on the amino acid transport has recently been observed also when protein synthesis was blocked by puromycin (Fritz and Knobil 1963 Carlin and Hechter 1964 Castles and Wool 1964) It has however also been reported that insulin and growth hormone stimulate the rate of protein synthesis in the isolated diaphragm under conditions when effects on membrane amino acid transport can be excluded (Wool and Krahl 1959 Manchester and Krahl 1959 Kostyo 1964) It might therefore be that insulin growth hormone FSH and possibly also other hormones which stimulate anabolic processes within their specific target organs have separate primary actions on the membrane transport system(s) for amino acids on the one hand and on some step(s) of the intracellular protein synthesis on the other hand Another possibility is however that the primary action of the hormone in question is localized to one type of receptor which then controls some aspect of cell metabolism essential for both amino acid transport and intracellular protein synthesis

We wish to thank the Endocrinology Study Section of the NIH for the generous gifts of NIH FSH S 1 NIH LH B-1 and NIH GH B-6 We also wish to thank Dr Bengt Johansson, Institute of Medical Biochemistry University of Goteborg for making the Kjeldahl analyses Insulin was kindly supplied by Dr Schlichtkrull Novo Research Institute Copenhagen and Pregnyl by Pharmacia Ltd Uppsala This investigation was supported by Grant RF 53 from U S Public Health Service Grants W 172 and Y 179 from the Swedish Medical Research Council and Grant 62 91 from the Swedish Cancer Society Valuable technical assistance was given by Miss Ewa Witt

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From the Department of Chemistry Karolinska Institutet, the Department of Internal Medicine Karolinska sjukhuset and King Gust[af V] Research Institute Stockholm, Sweden

Lipid Mobilization in Essential Fatty Acid Deficient Rats

A preliminary note

Prostaglandin and related factors 39

B

SUNE BERGSTROM and LARS A CARLSON

Prostaglandin E_1 (PGE_1) inhibits catecholamine stimulated mobilization of free fatty acids (FFA) from adipose tissue in anesthetized dogs (Bergstrom Carlson and Oro 1964 Steinberg *et al* 1964) In vitro PGE_1 inhibits the catecholamine stimulated as well as the basal release of glycerol and FFA from rat (Steinberg *et al* 1964) and human (Bergstrom and Carlson 1965 Carlson 1965) adipose tissue PGE_1 may thus have a physiologic role in the regulation of the rate of lipid mobilization from adipose tissue The recent finding that prostaglandins are synthesized from essential fatty acids (Bergstrom Danielsson and Samuelsson 1964 van Dorp *et al* 1964) prompted this preliminary study on lipid mobilization in essential fatty acid (EFA) deficient rats

EFA deficient rats and age matched controls were obtained from the Hormel Institute[1] in November 1964 They were kept on the proper EFA deficient and control diets in our laboratories for 1 to 3 weeks before use Two studies were done in the mornings with non fasting animals In the first epididymal adipose tissue from two control rats (mean weight 330 g) and two deficient rats (mean weight 170 g) were used In the second epididymal adipose tissue from 4 control rats (mean weight 450 g) and 4 deficient rats (mean weight 260 g) were used The adipose tissue was cut into pieces which were randomized between the incubation flasks containing Krebs Ringer bicarbonate buffer with 2 per cent human albumin and 0 1 per cent glucose and preincubated for about 30 min at room temperature Carlson 1965) Release of glycerol and FFA during incubation for 1 hr at 37 C was determined as described earlier with enzymatic and titrimetric methods respectively (Carlson 1965)

Table I shows that adipose tissue of the deficient rats was rather unresponsive to the inhibitory actions of PGE_1 and nicotinic acid (Carlson 1963 a on this preparation. The second study (Table II) also shows that PGE_1 had less inhibitory effect on the glycerol release in deficient than in control rats The deficient rats were at least as responsive to the stimulatory effect of noradrenaline on FFA and glycerol release as the controls (Table II) Both studies indicate that the rate of lipid mobilization was greater per gram adipose tissue incubated *in vitro* in the deficient rats The physiologic significance of this finding is unclear as these rats are leaner and thus presumably have a lesser amount of total adipose tissue However the value for the concentration of FFA in pooled plasma in the first study was 0 50 and 1 27 meq l for controls and deficient rats respectively and in the second study the FFA level for the 4 controls was 0 27 0 27 0 32 and 0 40 and for deficient rats 0 56 0 71 and 0 74 meq l (sample from

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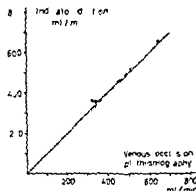


Fig. 1. Comparison between total blood flow values for the forearm and hand determined simultaneously with venous occlusion plethysmography and the dilution technique. Varying amounts of bradykinin were infused with the dye solution in order to increase the blood flow. The regression line (broken line, y = 1.14 x — 17, SD = 48, r = 0.97 *) does not deviate significantly from the line of identity (p > 0.1).

artery at rest and during light exercise it became important to determine the relationship between the infused dye solution and the initial blood flow. Oxygen saturation values for venous blood obtained before and during infusion of dye at this rate were found to be equal. Moreover the decrease in hemoglobin concentration indicated a simple addition of the infused solution to the initial blood flow within the range tested. No indication of active vasodilatation was found. The added infusion may have been accomodated by reduction of blood viscosity due to hemodilution (Rand and Lacombe 1964).

The dye began to reappear in the brachial artery after 20—40 sec. The concentration then rose linearly during the infusion and for 15—30 sec afterwards. It then fell off rapidly. The amount of recirculating dye could be accurately interpolated from the dye concentration in radial artery blood samples obtained immediately before and 15—20 sec after the infusion. Exact correction for recirculating dye in a particular blood sample requires that an amount be subtracted from the total dye concentration corresponding to the arterial background at one mean circulation time before the sample was obtained. The mean circulation times for the different sampling sites were determined from dye dilution curves recorded with a densitometer following a single dye injection into the brachial artery. The average values for each sampling site were used for the calculation of arterial background concentration.

A comparison was made between blood flow values determined for the forearm including the hand simultaneously with venous occlusion plethysmography and this indicator dilution method (Fig. 1). Varying amounts of bradykinin, a potent vasodilator in man (Fox *et al.* 1961), were infused with the dye solution in order to increase the blood flow. An acceptable agreement was found between the two methods.

A full account of this method will be published elsewhere.

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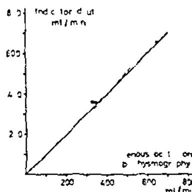


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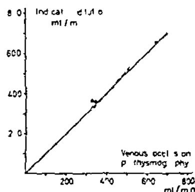


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